

A Study of the Seeds and Tubers of
Sandersonia aurantiaca (Hook.)

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ABSTRACT

The major proteins of *Sandersonia* seeds are salt soluble globulins visible as six subunits (bands) under SDS PAGE conditions. Electrophoretic characteristics also suggest that the globulin proteins in *Sandersonia* seeds resemble the vicilin sub-type. The major tuber proteins are water soluble albumins consisting of 4 subunits under SDS PAGE conditions. Differences in the solubility and subunit characteristics of seed and tuber protein disproved a hypothesis that they may be related, due to a similar life cycle function.

Through SDS PAGE and light microscopy (indirectly), the six seed subunits were found to form by 30 DAP, but not before 25 DAP. Between 30 and 60 DAP, the major seed proteins were found to accumulate, partially fulfilling the storage protein definition. Due to a deep dormancy mechanism, the seeds could not be germinated in significant quantities to demonstrate major protein degradation. The major seed proteins can only tentatively be defined as storage proteins.

The fate of the major tuber proteins was monitored, over the first six weeks after new season planting. No difference in the four-band (SDS PAGE) pattern was noted, during this time. The major tuber proteins were then monitored at three further developmental stages. Degradation of the major tuber proteins was observed to occur at the same time as seed formation and final daughter tuber formation. The major tuber proteins fulfil the storage protein definition regarding their degradation. Accumulation of the major protein is inferred through their presence at tuber maturity.

Three structural types of protein body were observed to occur. These accumulated differentially between tissues. SDS PAGE and TEM evidence indicate that the crystalloid type of protein body inclusions are absent from *Sandersonia* seeds, though the crystal and soft globoids are present, the former, in limited numbers, and the later in much larger numbers.

Thickening of the seed endosperm cell walls was observed and attributed possible functions of desiccation resistance and/or carbohydrate storage.

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ABBREVIATIONS

Bis	N, N'- Methylene - bis - acrylamide
BSA	Bovine Serum Albumin
CPE	Crystalloid Protein Extraction
DAP	Days After Pollination
KDa	Kilodalton
PAGE	Polyacrylamide Gel Electrophoresis
SDS	Sodium Dodecyl Sulphate
TEM	Transmission Electron Microscopy
TEMED	N-N-N'-N'-Tetra Methylethylenediamine
°C	Degrees Celcius
μ	Micro

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Sandersonia is an attractive ornamental and cut flower that has become commercially prominent in New Zealand recently. A native of South Africa, Sandersonia was brought to New Zealand over 83 years ago (Warren, 1988), but has only received significant attention for the last 16 years. This involved a number of large-scale commercial operations growing and exporting the cut flower stems. Scientific interest in the flower has also evolved around this industry, investigating optimal cultural practices for commercial production and flower senescence (Eason and De Vre, 1995).

Throughout this time though, no work has been published relating to the biochemical changes occurring during development of Sandersonia seeds and tubers. The scope of this thesis aims to investigate those areas of Sandersonia lifecycle.

1.2 What is Sandersonia?

1.2.1 Taxonomy

Sandersonia – *Sandersonia aurantiaca* (Hook.) – is a monotypic species in the Colchicaceae family. Presently, Sandersonia belongs to the tribe Iphigenieae, which also includes the close relatives *Gloriosa* and *Littonia* (Dahlgren *et al*, 1985). Previously these genera were grouped together as the tribe Glorioseae (Sterling, 1975). The Colchicaceae family is part of the Liliales, a monocotyledonous order. Sandersonia has the common names Christmas Bells, Chinese Lantern (Van Der Spuy, 1971), Golden Bells (Ijiro and Ogata 1999) and Golden Lily of the Valley.

1.2.2 Morphology

Sandersonia is an erect perennial herb with a subterranean stoloniferous corm (Dahlgren *et al*, 1971). A corm, by definition is a thickened underground stem, in which food is incorporated (and stored). In comparison a tuber is an enlarged short fleshy, underground stem such as that of the potato (Raven *et al*, 1992). Due to the difference in definition only relating to size, Sandersonia corms will henceforth be superficially referred to as tubers. This change in naming is necessary, so the limited amount of previous research, analogous to that presented in this thesis can be pooled and compared. The hypocotyls of yam are also considered tubers. For the purposes of this thesis, a tuber will be defined as a propagule containing a source of nitrogen and (to a lesser extent) sulphur for its own growth and development (Shewry, 1995).

Sandersonia mother tubers are fork shaped, with one ‘arm’ usually actively growing and the other ‘arm’ dormant. Extremely vigorous mother tubers may produce shoots from both ‘arms’ of the tuber. The adult stem and mother tuber die away at the end of the growth season. During the growing season two daughter tubers are formed, each with a single growing point (Brundell and Reyngoud, 1985). The two daughter tubers have the appearance of a single forked tuber but most authors describing the growth of daughter tubers, choose to identify the structure as two separate parts. While the two daughter tubers are still connected, one tends to be dominant.

In some instances, secondary tubers form on the growing points of the developing daughter tubers from 3 weeks after flowering (Brundell and Reyngoud, 1985). These secondary tubers can be round or fork shaped (Clark and Burge, 1997b) and can grow on both of the daughter tubers’ growth points. A fork shaped secondary tuber is a further development of the initially round secondary structure. Also called ‘grand daughter tubers’, ‘marbles’ or ‘buttons’ the secondary tubers can break off during lifting, ruining the daughter tuber (Clark and Burge, 1997a).

Secondary tuber formation occurs on all sizes of tubers and has been reported in tubers grown from seed. Secondary tubers are often too small for forcing (new season growth).

Mother tubers can be grown over three or more seasons, with a continual size increase each season. Each new season shoot is trimmed until only a small number of leaves remain, to support the needs of the growing tubers.

At maturity, *Sandersonia* has a tall wiry stem (sometimes greater than 1 m in height) with numerous leaves evenly dispersed. The height of an adult plant is directly proportional to the weight of the planted tuber (Brundell and Reynoud, 1985). Tall adult plants are produced by large 15-20 g tubers (the result of 3-4 seasons growth with pruning suppression of flowering).

Sandersonia has bright orange 'lantern-like' flowers (plate1 B) suspended on pedicels attached to the stem above each upper leaf (Eason and De Vre, 1995). The bisexual flowers develop as axillary buds on a monopodium, with fused tepals (Sterling, 1975).

After cross pollination *Sandersonia* produces seeds in a closed tricarpellate pistil (seedpod). The pistil is non-fleshy, containing endospermic (oily), wingless seeds (Sterling, 1975).

Though *Sandersonia* belongs to the same tribe as *Gloriosa* and *Littonia*, it differs from these genera in morphology. Where as *Sandersonia* is an erect herb, *Littonia* is a twining herb and *Gloriosa* is a vine with branches up to 150cm long. *Sandersonia* has fused tepals to form the characteristic lantern shape, whereas *Gloriosa* are free, only forming a cover for the reproductive organs, which forms after pollination (Dahlgren *et al*, 1985).

Sandersonia relates to *Gloriosa* and *Littonia* by the production of a corm (tuber). Each of the three genera produces stoloniferous corms containing the alkaloid colchicine.

1.2.3 Habitat

Sandersonia is a native plant of South Africa and grows in the highlands of, Pondoland (Van der Spuy, 1971), Natal, Transvaal, the Kingdom of Swaziland and the Eastern Cape. It grows at altitude, 600-2000 m in areas of high summer and low winter rainfall, and with low soil fertility (Clark, 1997).

Sandersonia relatives *Gloriosa* and *Littonia* are tropical African-Asian and South African respectively, though two species of *Littonia* have been found in Arabia (Sterling, 1975).

In South Africa *Sandersonia* is rare in the wild and is classed as an endangered species. *Sandersonia* is now a protected species, its' harvest being illegal in the wild (Warren, 1988).

1.3 Growth and Culture Conditions

Sandersonia can be reproduced by tuber or seed, the later with varying success. The tubers can be planted in well draining soil or potted, soil-less media. A light friable media is used by growers for lifting the tubers without breakage. A slightly acidic medium (pH 5.0-6.0) is preferred by the plant. Higher pHs can cause leaf chlorosis and browning (Clark, 1994¹). A low-medium amount of nutrient addition to the soil is required by the plant (Clark and Burge, 1999a). Adding high amounts of additional nutrients to the media can cause the plant to form secondary tubers at a higher rate (Clark and Burge, 1999b). The new seasons' shoots emerge 4-5 weeks after tuber planting and flowering begins another 4 weeks after that. Approximately 7-8 weeks after sprouting the stems die back, and the daughter tuber becomes dormant.

Sandersonia tubers grown outdoors in New Zealand are dormant for up to 7 months (Clark, 1995). Plants die down in April and emerge in October, flowering

in late November-early December, with stem lengths of 50-70 cm (Brundell and Reyngoud, 1985). Winter temperatures in New Zealand are similar to those of the natural South African habitat. The 4 to 6 month winter temperatures of the Natal Highlands naturally overcome tuber dormancy (Clark, 1995).

When grown indoors, dormant tubers can be lifted and chilled at 4°C, breaking the dormancy in a time faster (8-12 weeks, Clark 1994¹) than what naturally happens in the soil in New Zealand (up to 7 months). After the dormancy has been broken, the tubers are ready for new season planting.

Reproducing *Sandersonia* from seed is more difficult, due to a deep dormancy mechanism. A typical stem produces 8-12 flowers, each bearing 50-70 seeds, though seed germination is often less than 20% in the first year (Clark, 1994¹). Growers overcome the deep dormancy by sowing seeds outdoors to provide the chilling and leaching required. 1-2 g tubers are produced by seeds within the first year (Clark, 1994). Previous researchers have found it difficult to germinate *Sandersonia* seeds in vitro, due to this deep dormancy mechanism (Ijiro and Ogata, 2000). Once flowering, adult plants have been produced, whether by seed or tuber, the flowers must be pollinated by hand for good seed set (Clark, 1994¹).

1.4 *Sandersonia* History in New Zealand

An informal historical account of the introduction of *Sandersonia* to New Zealand (Warren, 1988), states that Donald Ross, of Hawera (North Island, New Zealand) brought several tubers home after a trip to South Africa. These tubers were planted and gifted to friends. The first New Zealand grown flowers for sale were produced by Dave Doig of Tauranga.

¹ See Appendix 2.

These flowers were produced by tubers, grown from the original tubers Donald Ross imported. Due to trade restrictions with South Africa, the majority of New Zealand's Sandersonia tuber stock originates from those early tubers, and not from any official or commercial agreements.

1.5 Sandersonia: Commercial Importance As A Cut Flower

Internationally, Sandersonia is important as a cut flower and potted ornamental plant. In 1995 Sandersonia was ranked as the country's third most valuable cutflower export (Brooking *et al*, 1997). From New Zealand, Sandersonia is exported to Japan (Warren, 1988)

New Zealand has a small number of progressive growers that are responsible for the majority of Sandersonia stems exported. These growers¹ advertise via the internet, the ability to produce tubers and stems year-round (indoors). One major grower of Sandersonia, 'Bloomz' works in collaboration with the New Zealand Institute for Crop and Food Research Limited-a public sector company. Therefore, the majority of journal publications of research on Sandersonia involve solving problems related to commercial production.

1.6 Growth and Culture for Commercial Production

Because Sandersonia is a valuable floriculture crop, the majority of research within the last 8 years has focused directly on finding the optimal culture conditions for plant growth and commercial benefit. Experimentation has focused on finding culture conditions, which optimise several plant variables (flower number per stem, stem length, daughter tuber weight), and reducing the occurrence of undesirable traits (secondary tuber production, floral senescence)(Clark, 1997a, 1999b, and Eason and De Vre, 1995). Also it has been found that long stems are often

¹ See Appendix 2

produced at the detriment of daughter tuber growth (Ijiro and Ogata, 2000). Therefore, there must be a prioritisation of the desired features.

Previous commercially focused research has shown that:

1. Growers can produce flowering stems all year round indoors, with proper storage temperatures (4°C), which prevent flowering until required. Storage at 4°C for 90-120 days is required for rapid and even shoot emergence (Clark, 1995).
2. Growers can cut the forked daughter tuber in half to get even (non-dominant) shoot emergence from the two resulting daughter tubers, though two small stems are produced rather than one long stem (Clark, 1994).
3. Daughter tuber growth and secondary tuber formation are more prevalent at certain times of year. Later planting dates produce less secondary tubers and lower daughter tuber growth weights. Environmental factors (temperature, day length) may be responsible for changes noticed at later planting dates (Clark and Burge, 1997a).
4. High plant density together with pruning (above the second set of leaves) significantly decreases the formation of secondary tubers (Clark and Burge, 1997b). This study also found that secondary tuber initiation can begin up to seven weeks after flowering, previously Brundell and Reyngoud (1985) described secondary tuber development as occurring three weeks after flowering.
5. High crop plant density and low nutrient application rate, the rate of secondary tuber formation was reduced to 38%. Previously, a low plant density and high nutrient rate produced a secondary tuber formation rate of 79%. They speculate that the low rate of secondary tuber formation in the high density crops was due to increased interplant shading. (Clark and Burge, 1999b).

6. Media and nutrition affect the levels of tuber russetting. Russetting is a process where the tuber surface cuticle becomes cracked or blemished with undesirable colours, resulting in the tuber becoming non-saleable. Clark and Burge (2000) found that in peat:pumice or soil, russetting is minimised by using a low nutrient rate. In bark, a higher nutrient rate can be applied, with the same low level of russetting.
7. Applying a high nutrient rate to crops of *Sandersonia* tubers does not increase the stem length, number of flowers per stem, or vase life of stems (Clark and Burge, 2000).
8. Low temperature (15-17°C) and high irradiance are required to produce large 4th generation tubers (Brooking *et al*, 1997). If high quality (long) stems are required at the risk of increased development of secondary tubers, higher day/night temperatures (30/24°C) are required. If more flowers, increased flower stalk elongation and increased daughter tuber formation are required, day/night temperatures of 24/17°C are necessary (Ijiro & Ogata, 2000). Both research groups have found that secondary tuber formation is at a minimum at low temperatures (15-18°C).
9. *Sandersonia* floral senescence is ethylene insensitive (Eason and De Vre, 1995).

1.7 Storage Proteins in Seeds

Previous research on *Sandersonia* biochemistry has been conducted on the problem of floral senescence (tepale protein content, Eason and De Vre, 1995) and the occurrence of colchicine production in the tuber (Finnie and Van Staden, 1992). No research relating to the storage protein biochemistry of *Sandersonia* seeds and tubers has ever been published. The same situation applies for *Sandersonia* closest relatives *Gloriosa* and *Littonia*. Nevertheless, a lot of published research investigating the seed and tuber proteins of other species provides an analogous

background for the techniques involved and the results that could be reasonable expected (involving seeds' and tubers' physical characteristics).

The following literature review discusses the observations relating to seed storage-protein development of other plant species.

Plants deposit and mobilise protein reserves during several different stages of development. The most well known example is the deposition of seed storage proteins in the endosperm and embryo, which are mobilised to provide nutrients during germination (Bevan *et al*, 1993). Seed storage proteins can be defined as “any protein accumulated in significant quantities in the developing seed which on germination is rapidly hydrolysed to provide a source of reduced nitrogen for the early stages of seedling growth” (Higgins, 1984). Spencer (1984) gives the same definition, but adds that they (storage proteins) should “occur only in the seed” and that this source of reduced nitrogen commonly contains a high proportion of amides, glutamine and/or asparagines. In mature seeds, storage protein accumulates in the endosperm tissue. At cellular level, the protein accumulates in organelles called protein bodies and in some rare instances storage proteins accumulate in the cytoplasm (Higgins, 1984). Seeds commonly contain three types of protein; structural, metabolically active ('house keeping') and storage (Marcone *et al*, 1998). In seeds, storage proteins accumulate to high quantities, but a low number of different protein species are present. In comparison, 'housekeeping proteins', essential for the maintenance of normal cell metabolism, accumulate to relatively low levels, but represent a diverse range of protein species (Higgins, 1984).

1.7.1 Worldwide Importance of Storage Proteins

The seeds of crop plants provide a large proportion of the protein consumed in the human diet. Approximately 70% of the edible protein produced in the world comes from seeds (Spencer and Higgins, 1979). The protein from crop plants' seeds are very important for the diet of livestock, such as poultry and pigs (Spencer, 1984), and as industrial raw materials (Shewry, 1993).

For this reason, some aspects of seed proteins, their content and development, gained most research attention in the late 1970s.

Because seed proteins are important economically, the majority of research on this topic related to the development and constituents of the seed proteins of legumes and cereals. Historically, with the promised tools of molecular biology, the long-term goal of most research efforts was the modification of seed proteins to suit monogastric animals (humans, pigs and poultry) (Spencer, 1984). The ‘problem areas’ related to edible seed proteins were the imbalances in the 10 essential amino acids. Proteins of cereal seeds are typically deficient in lysine, tryptophan and threonine, while leguminous seeds are typically deficient in methionine and cysteine (Spencer and Higgins, 1979). Most research on the topic was concerned with the feasibility of modifying the protein contents, to possibly incorporate the missing constituents, without disrupting the seeds’ germination efficiency.

1.7.2 Classification of Seed Storage Protein Types

The seed storage proteins can be divided into four groups based on differing solubility. First described by Osborne in 1924 (Bewley and Black, 1978), the four groups are: albumins, globulins, prolamins and glutelins, each being soluble in water, dilute salt solution, aqueous alcohol, and acid or alkali solutions, respectively. With some exceptions, the different protein groups are each found in different plant groups (as the main storage protein present). The legumes and other dicotyledonous plants contain globulins and the monocotyledonous (cereal and crop) plants contain prolamins and glutelins as their major storage proteins. An exception is oats which contains a globulin storage protein (Higgins, 1984). Although these plant groups contain a major protein class that defines them, individual plant species still contain some of the other protein classes at low levels (Bewley and Black, 1978). Within each of the four solubility groups there is considerable heterogeneity.

Within the four solubility groups there is further subdivision, based on physical properties (sedimentation coefficients, precipitation with ammonium sulphate, heat coagulability and electrophoretic activity).

The globulins can be divided into legumin-like proteins and vicilin-like proteins, which can each, be split into constituent subunits. The legumin-like proteins are also found in some non-leguminous plants such as *Prunus* spp and *Brassica* spp (Bewley and Black, 1978).

The legumin-like proteins have a molecular mass of ~ 360,000 Da (11-12S), consisting of six similar subunits (~ 60,000 Da each), each containing an acidic and basic polypeptide (~ 40,000 and 20,000 Da each, respectively) covalently linked through a disulphide bond (Spencer 1984). Another researcher has found that some legumins contain subunits in the narrow ranges of 20,000-24,000 Da and 30,000-37,000 Da. There may be three different types (size) of subunit, and different numbers of each type, but as a whole, legumins are considered more homogeneous in their structure, some containing only one protein (Bewley and Black, 1978).

The vicilin-like proteins have a molecular mass of ~ 180,000-200,000 Da (7-9S) with a more complex trimeric subunit structure that does not involve disulphide bonds (Spencer, 1984), instead utilising non-covalent forces (hydrogen bonds and hydrophobic interactions). The vicilin-like protein subunits have a broader size range, 23,000-56,000 Da, and appear to be more heterogeneous, consisting of more than one protein. Five vicilin-like protein subunits have been described, each differing in polypeptide sequence (Bewley and Black, 1978).

Within one plant species, the two different types of globulin protein fraction may be present (together with some of the other solubility type proteins). And between two different plant species the same globulin fraction type may be different at the holoprotein and polypeptide level (Spencer and Higgins, 1979).

The prolamins are present as monomers or small aggregates containing 3 subunits. In wheat prolamins can form polymers linked by disulphide bonds (Shewry, 1995).

The glutelins form large disulphide bond aggregates, which are more heterogenous than any of the other storage proteins, with as many as 15 polypeptides, with molecular weights of 11,000-133,000 Da.

The albumins (2S proteins) were first thought to be enzymes and metabolic proteins, but have been demonstrated as being storage proteins in Castor Bean (*Ricinus communis* L.) constituting 40% of the total seed protein. Albumins have gained less research attention than the other 3 solubility classes of proteins, but are considered to be composed of subunits (Youle and Huang, 1978). Albumins are compact globular proteins made up of large and small subunits, that may or may not be connected by interchain disulphide bonds (Shewry, 1995).

The solubility characteristics of each of the four protein groups is determined by the individual subunits primary structure and the bond type between subunits (Shewry, 1993).

Two other characterised groups of seed storage proteins, not discussed in relation to the scheme of Osborne are the crystalloid and globoid inclusions of protein bodies.

The insoluble crystalloid proteins of some plant species are initially similar to globulins (partially soluble in salt solutions), but are only completely dissolved in detergent (sodium dodecyl sulphate) solutions and urea. Under electrophoresis the crystalloids are tetramers, shown to be composed of two identical subunits each comprising of two proteins linked by disulphide bridges (covalent forces). Most crystalloids are non-glycosylated and have similar solubility and size (molecular weight) characteristics but are immunologically different (Gifford 1987). Examples of crystalloids initially described as globulins are the 11S globulin 'edestin' of Hempseed (*Cannabis sativa*) (reviewed in Bewley and Black, 1978; Pernollet, 1978) and the 11S crystalloid protein inclusions of Castor Bean (*Ricinus communis*) in Gifford *et al*, 1982 and Youle & Huang 1978). Where the crystalloids fit into the different solubility classes of storage proteins is still an area of debate.

The globoid inclusions (crystal type) were thought to be different from the crystalloids in that the proteins present are commonly enzymes, but there is some evidence that they are globulins (Bewley and Black, 1978).

1.7.3 Overview of Seed Storage Protein Formation

The rough endoplasmic reticulum (RER) is responsible for the synthesis of storage proteins (Higgins, 1984). Proteins are synthesized as precursors on the RER (a membrane bound polysome), containing a signal sequence that specifies their transport path during production. The rate of protein formation is proportional to the levels of mRNAs (Meinke *et al*, 1981). The synthesized polypeptides (with signal sequences attached) are then directed to the RER lumen. Protein folding and subunit binding take place in the RER lumen. Before leaving the RER, the signal sequences are cotranslationally removed and in some instances, there are cotranslational protein glycosylations. Newly synthesized storage protein accumulates transiently within the RER prior to transport.

Between the major plant groups (legumes, cereals, dicots) there are differences in the final stages of deposition. In some instances, the proteins are transported to the golgi body, where further modification (post-translational glycosylation or unglycosylation) takes place. The proteins are packed into vesicles which transport the proteins to the final deposition, into vacuoles which form protein bodies-an example is the legume globulin legumin of field beans (*Vicia faba* L. var. *minor*) (Muntz *et al*, 1993). In other instances, proteins are deposited directly into the cytoplasm, and in some cases the protein is packaged into vesicles of RER origin. Within cereals (barley and wheat) two final-formation pathways have been suggested. The globulins are deposited into protein bodies via the golgi bodies and subsequent deposition (via vesicle) into a vacuole which fragments to form a protein body. In comparison the prolamins (of barley and wheat) are thought to accumulate in the RER lumen before a small part of the RER shears away to form the protein body (Shewry, 1993).

The 11S globulins and most prolamins are generally considered unglycosylated. The 7S globulins are glycosylated-an oligosacchiride side chain added to the nascent polypeptides. These post-translational modifications may be used in directing the protein to the intended cellular storage site (Higgins, 1984), though it is still unclear whether the post-translational modifications are essential for protein sorting, as a default pathway may operate-sorting unmodified proteins (Harris *et al*, 1993).

Protein accumulation in legumes shows a sigmoidal pattern. The different storage protein types have differential rates of accumulation: the 11S and 7S globulins accumulate at different rates. These differences may be based on mRNAs and the rate of synthesis (Higgins, 1984), Yamagata *et al* (1982) found, using rice that the formation of protein subunits coincided with increasing levels of cellular mRNAs, possibly also confirming Higgins observation.

This summary should be considered only a basic overview of the processes involved in storage protein formation and deposition, for each of the four solubility classes. It should be noted that, within each of these classes, there still remains some debate over the regulation of final protein deposition, the mechanisms of which are extremely complex.

1.7.4 Physiological Role of Seed Storage Proteins During Early Seedling Growth

During germination seeds pass through several stages which are the reverse of those occurring during seed maturation-imbibition, and rehydration (Werker, 1997). In cereals, enzymes (peptidases and alpha amylases) synthesized in the outer aleurone layer or the embryo itself, breakdown the dead endosperm tissue, mobilising protein used for embryo growth during germination (Harris *et al*, 1993).

Seed storage proteins are a source of reduced nitrogen, and in some cases, minerals (as phytin), providing ‘building blocks’ for the successful germination of the mature seed. Phytin is the major source of phosphate and macronutrient mineral elements in seeds. Phytin is an insoluble mixed potassium, magnesium and calcium

salt of myo-inositol hexaphosphoric acid (phytic acid) and is found in the globoid inclusions of protein bodies of dicotyledonous seeds and the aleurone grains of cereals. The total amounts of cellular phytin differ between plant species (Bewley and Black, 1978).

In the germinating seed, storage proteins provide reduced nitrogen for the construction of enzymes to increase cell metabolism, structural protein formation for use in meristematic cell division (Werker, 1997) and organogenesis, and 'house keeping' proteins for metabolically active cells (Higgins, 1984).

1.7.5 Previous Research on Seed Storage Protein Development

Previous research on the development of seed storage proteins can be split into two groups: the earlier research into storage proteins of crop related plants (cereals and legumes) and the later research into the storage proteins of more diverse plants (dicotyledons, conifers). Due to the greater history of study of crop related seed storage proteins this group has been examined in greater depth. The results of these studies have been used to generalise the modes of formation of storage proteins of different plant groups (previously described in sections 1.7-1.7.4).

Research into the electrophoretic characteristics of storage proteins from a diverse range of plants (other than the crop plant groups-legumes and cereals) has netted some interesting results:

1. Luthe (1992) analysed the SDS PAGE characteristics of mature seed representatives of each of the 58 dicotyledonous orders. It was found that most of the representatives contained (bands representing the subunits of) legumin-like proteins as their major storage protein or in some quantity, while others had a mixture of legumin- and vicilin-like proteins. Only a small number had vicilin-like proteins as their major storage protein class. From this study the author concluded that legumin-like proteins are ubiquitous throughout the plant kingdom.

2. Marcone *et al* (1998) analysed the seed globulins of 21 food-related monocotyledonous and dicotyledonous plants. It was found that all of those globulins were of the 11S legumin-like type, with non-dissociated protein molecular masses of 300-370 KDa. Interestingly, the majority of the globulin representatives had subunits held together by non-covalent forces and only a small number had subunits bound by covalent (disulphide) forces. The small number of proteins that did have covalent forces yielded more SDS PAGE bands when treated with a reducing agent (mercaptoethanol), whereas the subunits held together with non-covalent forces yielded no more SDS PAGE bands when treated with a reducing agent.

3. Gifford (1987) analysed the SDS PAGE characteristics of 9 *Pinus* species' seeds. It was found that all the *Pinus* species examined, contained insoluble non-glycosylated crystalloids as some percentage of their total mature seed protein content. Eight out of 9 *Pinus* species contained crystalloids as greater than 50% of their total mature seed protein content. Under non-dissociating conditions (-SDS) the whole proteins produced gel bands of 51-55 KDa, under dissociating conditions (+SDS) the proteins separated into 2 subunit groups 31.5-35 KDa and 21-22.5 KDa. The crystalloid inclusions are not restricted to coniferous plants.

Having discussed the large amount of heterogeneity relating to storage protein form, it is still worth noting that the storage protein groups, of evolutionarily diverse groups of plants, still show striking homology with regards to structure at different levels of protein organisation. With this in mind, seed storage protein structure has been suggested as a tool for evolutionary study and taxonomic purposes (Luthe, 1992). The sequence homology between equivalent storage proteins from diverse phylogenetic origin suggests a common genetic ancestry, and may reflect the conservation of those sites could also be essential for the physiological role of germination (Higgins, 1984).

As stated in section 1.7.1, the majority of research into the nature and rate of formation of seed storage proteins has been conducted on economically important

cereal crops. As electrophoretic techniques have advanced, the understanding of storage protein structure has increased. Along with this understanding there has been a race to discover the nature and structure of seed storage proteins of more diverse plant groups such as food related dicots and monocots (Marcone *et al*, 1998), botanical order representatives of the dicots (Luthe, 1992), and conifer species (Gifford, 1987). Nevertheless there still remains a lack of research into the seed storage proteins of monocotyledonous angiosperms (of which *Sandersonia* is a member).

1.8 Storage Proteins in Tubers

After the breakage of seasonal dormancy, most tubers produce a shoot(s), which is intended to grow until reproductive maturity. The tuber supports the growth of the shoot until enough leaves are produced, for photosynthetic self-sufficiency. To enable this to occur, tubers accumulate nutrient reserves in the form of storage proteins

1.8.1 Tuber Storage Proteins and Previous Research

Like the seed storage proteins, tuber storage proteins are synthesised on the rough endoplasmic reticulum and either deposited within the ER lumen or transported to a vacuole for protein body formation.

The majority of previous research, relating to the nature of tuber storage proteins, has only been conducted on the most commercially important tubers.

The commercially important tubers (potato, sweet potato, taro and yam) are of diverse taxonomic origin, consequently their respective storage proteins show little homology (Shewry, 1995).

Potato (*Solanum tuberosum* Solanaceae, a dicot) tubers have a major storage protein family – Patatin, a family of 40 KDa glycoproteins, which account for 40% of the soluble tuber protein. 12-15 immunologically identical patatin polypeptides are present in different potato cultivars. Patatin subunits display a charge

heterogeneity, considerable enough to cause some previous researchers to conclude that potato has no one major storage protein (Park *et al*, 1983). Patatins are coded for by two sub-families of mRNAs (class 1 and 2 patatins). Class 1 patatin mRNAs encode most tuber patatins, while class 2 patatin mRNAs encode root expressed patatins and some tuber patatins. Patatin has intrinsic biological activity as a lipid acyl hydrolase and acyl transferase. The significance of this activity in relation to its storage function is unknown (Shewry, 1995). The soluble storage proteins of potato were previously called ‘tuberins’ (70% tuberin and 30 % tuberinin, each differing in their isoelectric points, Harris (ed), 1978) by one research group, but are now considered the same as the patatins (Park *et al*, 1983). The protein solubility types’ present in potato storage proteins has been a controversial issue. Different researchers claim a variety of albumin; globulin; prolamin; glutelin ratios (Desborough, 1985).

Sweet Potato (*Ipomoea batatas* Convolvulaceae, a dicot) tubers are derived from swollen roots. The main storage protein is sporamin (a globulin), which accounts for 60-80% of the total soluble protein. There are two sub-types of sporamin, sporamin A (31 Kda) and sporamin B (22 KDa) (Conlan *et al*, 1998), which are encoded for by two different cDNAs (A and B). In the mature tuber the sub-types are present in a ratio of 2:1, for A and B. Under field conditions, sporamins expression is limited to the tuber, but can be induced in vegetative tissues under special conditions. Sporamin is in the Kunitz family of trypsin inhibitors (Shewry, 1995).

Taro (*Colocasia esculenta* Araceae, a monocot) contains several groups of proteins, that are largely tuber specific, but the presence of true storage proteins is yet to be confirmed. Those tuber specific proteins consist of an albumin and two globulins. The albumin protein accounts for 11% of the total soluble protein and consists of 50 KDa protein, made up of 8.3 KDa subunits linked by interchain disulphide bonds and is present in one major and two minor forms. The two globulin protein groups are 14 and 22 KDa. The 14 KDa globulins are related to the sweet protein curculin from fruits of *Curatigo latifolia*. The 22 KDa globulins are

related to the Kunitz family of trypsin inhibitors and the taste modifying protein, miraculin from fruits of *Richardella dulcifera* (Shewry, 1995).

Yam (*Dioscorea* spp. Dioscoraceae, a monocot) has seven important crop species. *Dioscorea* tubers contain the major storage protein dioscorin (31 KDa), which accounts for 80% of the soluble protein. Dioscorins are made up of two classes of subunits, possibly containing intra-chain disulphide bonds. Though dioscorin has a significant degree of sequence similarity to alpha-carbonic anhydrases, it has not yet been demonstrated to exhibit any enzymatic activity (Conlan *et al*, 1998)

1.8.2 General Features of Tuber Storage Proteins

Shewry (1995) has made the following conclusions relating to tuber storage proteins, based on the limited information gathered from the three tuber types (potato, sweet potato and yams), with true storage proteins,

1. There is no apparent relationship between the major storage proteins of the three species. Each is derived from protein families, and may have different secondary activity.
2. Some tuber storage proteins (potato and sweet potato) are not only tuber specific, occurring also in tissues other than the tubers (only with proper initiation) (Paiva *et al*, 1983). This contrasts with seed storage proteins, which are not synthesised in any other tissue.
3. Despite their differences' sporamin, patatin and dioscorin are each encoded by multigene families, each with two subfamilies.

Due to this apparent lack of knowledge relating to tuber storage proteins it was of interest to examine any tuber storage proteins present in *Sandersonia*.

1.9 Protein Bodies

Storage proteins are synthesized in large amounts (up to 40% of the dry weight of soybean) and consequently, need to be stored in a highly concentrated form, in storage compartments separated from the normal metabolic processes of the cell. This is achieved by the different solubility properties and the deposition into protein bodies (Shewry, 1993).

Protein bodies had previously been termed aleurone grains, due to their (rare) occurrence in the aleurone layer of some monocot seeds, but this name has been abandoned, as the organelles were found to appear in tissues other than that of the aleurone layer (Lott, 1981).

Protein bodies are small, round organelles surrounded by a single membrane of tonoplast origin from the ER and range in size from 1-25 micrometers in diameter (Pernollet, 1978). Within the membrane is a homogenous proteinaceous matrix material that may be composed of more than one type of storage protein (Lott, 1981). Protein bodies commonly contain inclusions within the matrix material-crystalloids and globoids, of which globoids are the most common.

Globoids are either electron-dense black crystals or electron-transparent white 'soft globoids' (Lott, 1981). There is still a degree of debate over the formation and function of the soft globoids. Electron dense globoids (and soft globoids, according to some authors) contain phytins (discussed in section 1.7.4): potassium, magnesium and calcium salts of phytic acid (myo-inositol hexaphosphoric acid) (Bewley and Black, 1984). Phytin is the major storage form of phosphate and macronutrient mineral elements in seeds. Globoids have a boundary region that may be a membrane, separating them from the matrix material (Pernollet, 1978). Other authors describe soft globoids as water or fluid stores, or even air pockets (Werker, 1997).

Crystalloid proteins are ordered, partly crystalline, proteinaceous structures which lack a membranous boundary layer (Pernollet, 1978). Crystalloid proteins of some species are described as globular-like (Bewley and Black, 1978) and non-

globular-like structures (Gifford, 1987). They vary in shape, size and number. In any plane, crystalloid proteins may be angular, irregular or rounded (Lott, 1978). Where globoid and crystalloid proteins occur in the same protein body, crystalloids are considered the main protein deposits and crystal globoids are considered non-proteinaceous mineral stores (Pernollet, 1978).

Protein bodies may also contain ‘druse’ crystals of calcium oxalate as single large crystals or aggregations of smaller crystals (Lott, 1978).

Protein bodies are mostly found in the triploid-endosperm storage tissue and the embryo (in small numbers) of monocotyledonous seeds, but are absent from the seed coat (aleurone layer), which contains the constituents for enzyme secretion during germination. Dicotyledonous seeds have no special storage tissue, though protein bodies are found in the cotyledons and the embryo. The characteristics (size and constituents) of protein bodies may vary in one seed and their distributions between cells can be unequal. Lott (1978) proposed 11 models of protein body organisation involving different combinations of inclusion bodies, and regards the differences in protein bodies between families as a useful tool in the study of systematics.

Examining the types of protein bodies and inclusions present (if any) in *Sandersonia* seeds, via TEM, should be considered a useful addition and possible verifier to biochemical analysis obtained through electrophoresis (SDS PAGE).

1.10 Aims and Objectives

Recent studies have almost established the optimal culture conditions for tuber growth. However many unresolved problems particularly secondary tuber formation and low seed germination rate remain. An avenue of research that has not yet been explored, which may help in the understanding of *Sandersonia* culture, is the biochemistry of *Sandersonia* tubers and seeds.

With that in mind, further research into optimising *Sandersonia* cut flower production needs to examine biochemical aspects of *Sandersonia* development. The

aim of this thesis was to increase the understanding of the biochemical aspects of *Sandersonia* development. In particular, a better understanding of the developmental biochemistry of *Sandersonia* storage organs may provide a useful foundation of further research and may also lead to an increase in commercial cut flower production.

The specific objectives of this thesis research are:

1. To produce a crop of adult plants, from which seeds could be harvested.
2. To analyse the mature major seed proteins through SDS PAGE (dissociating electrophoresis), comparing the results to previous, analogous research.
3. To carry out germination tests, to test for seed viability.
4. To observe the formation of the major seed proteins from the date of pollination, to maturity via SDS PAGE, light microscopy and transmission electron microscopy (TEM).
5. To establish whether the major tuber proteins have the same electrophoretic characteristics as the major seed proteins via SDS PAGE.
6. To observe major tuber protein degradation during shoot development, in the new growing season.

CHAPTER 2

METHODS AND MATERIALS

2. 1 Sources of Plant Materials

A small number of flowers were gifted to the writer by Dr D Leung. Eighteen small first generation tubers were purchased from Oderings Nurseries (CHCH) LTD (distributed by Fiesta Flower Bulbs) and had already sprouted shoots at the date of purchase. These tubers were the first crop planted (known as crop 0). Tubers were also purchased from Bloomz NZ LTD and appeared bigger in size and healthier than the previously sourced tubers. These tubers were large third generation tubers. These tubers made up the final three crops (known as crops 1,2 and 3). Only 2 lots of tubers were purchased from Bloomz NZ LTD (crops 1 and 2). At the end of the flowering stage, the tubers produced by crop 1 were excavated and chilled for 12 weeks according to Clark (1995). After this time, the tubers were replanted to create crop 3. Due to the limited research time, only 3 crops were planted. After harvest, the 2nd crops' tubers were put in storage at 4°C and remained there during the remaining term of research.

2. 2 Planting Conditions and Media

All tubers were planted in a stratified medium consisting of two layers, a top layer of vermiculite and a lower layer of potting mix (see appendix 1). The tubers were planted at the intersection of the two layers. Large (15 cm diameter) polythene pots with draining holes were used. Tubers were watered twice weekly depending on season and experiment, as described in the method section (2.10).

Crop 0 tubers were watered 120 mL twice weekly, crops 1, 2 and 3 were watered 150->250 mL twice weekly.

2. 3 Glass House and Incubator Room Conditions

Crop 0 was planted in a growth room in an attempt to establish a crop of mature plants. The room was artificially lit using '840' colour fluorescent lights for a 16-hour photoperiod. The artificial photoperiod was conducted at night. The temperature was maintained at 22°C, though the room was not humidity controlled. Planter pots sat on raised 0.5m mesh stands.

Tuber crops 1, 2 and 3 were grown in a semi-controlled glasshouse. The glass ceiling let more light in than the semi-transparent plastic walls. Within the glasshouse, the plants were located next to a west-facing wall. The plants only experienced full photon flux density when the sun was directly overhead, between 11 am to 2 pm during mid summer. The glasshouse was semi-temperature controlled. When temperatures descended below 21°C heating began. When summer temperatures exceeded 26°C, sensor controlled ceiling vents opened, but temperatures above 30°C were easily achieved when the cooling effects of the vents were overwhelmed by midday radiant heat. Planter pots were placed on raised (1m) concrete benches. Bamboo and wood stakes were used to support adult plants, which could not support their own weight. When adult plants grew to their full height of approximately 1.5 m a permanent construct was used. This was constructed of long vertical stakes anchored to the walls by string. Crops 1, 2 and 3 were grown with exposure to full sunlight at midday. The final crop (3) was grown with a retractable sunshade in place, though this was removed at the start of the flowering stage.

2. 4 Germination Tests

Mature *Sandersonia* seeds from crop 2 were soaked in distilled water overnight. Then they were placed on one sheet of germination paper moistened with autoclaved distilled water in petri dishes sealed with plastic film. The petri dishes were incubated in darkness at 24°C. An earlier germination test was carried by placing mature seeds on moist filter paper in sealed plastic petri dishes. The

dishes were incubated in a 22°C 16 hour photoperiod room with a plastic container covering.

2. 5 Seed Dissection (Embryo Detection)

Seeds were dissected by scalpel and forceps on a petri dish lined with moist filter paper. The presence or absence of embryos was noted using a stereo microscope.

2. 6 Seed Proteins

Test seeds (gifted by Dr Leung) were ground in protein extraction buffer using a small pre chilled mortar and pestle. The type of extraction buffer (1-5 mL) used depended on the nature of the experiment. The homogenate was chilled on ice for 30 minutes before being centrifuged at 15,000 rpm for five minutes at 4°C. The resulting supernatant was collected and stored at -20°C.

A rotary grinding mill was used in some instances to grind mature, hardened seeds, though a mortar and pestle was still required after the grinding process to break down large endosperm pieces. Extracted seed protein samples were either used directly in the Bradford Assay (Bradford, 1976) or prepared for Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis. Protein samples (160µL each) were mixed with 40 µL SDS PAGE sample buffer before being heated in a 100°C water bath for five minutes.

2. 7 Tuber Proteins

Tuber samples were removed from storage at -80°C and weighed. All samples intended for comparison in the same SDS PAGE gel were cut to a similar size by scalpel and their weight roughly standardised. Tuber tissue samples were ground in pre-chilled mortars with 1mL extraction buffer. Extracted tuber protein samples

were stored in labelled eppendorf tubes at -20°C. Extracted tuber protein samples were standardised after the Bradford assay (2. 9. 1). For SDS PAGE, 160 µL tuber protein extracts were mixed with 40µL SDS PAGE sample buffer and boiled for 5 minutes in a 100 °C water bath.

2. 8 SDS PAGE

All SDS PAGE gels were cast and run using the BIO RAD Mini-PROTEAN® II Dual Slab Cell System and Laemmli gel and buffer systems (Laemmli, 1970). 5µL - 15µL samples of pre-fixed protein were loaded into wells in the construct. Gels were electrophorised at a constant voltage (200 volts) for 42-45 minutes. The gels were then stained and destained according to the BIO RAD Mini-PROTEAN® laboratory manual. All gel preparations and reagents used are listed in appendix 1.

2. 9 Protein Determination

2. 9. 1 Bradford Assay

The protein quantitation procedure used was essentially that reported in Bradford (1976). One hundred microlitre samples of extracted protein were mixed with 1mL of Bradford reagent (appendix 1). After one minute standing absorbance was read at 595nm was read using a Novaspec II spectrophotometer. The spectrophotometer was previously 'set to zero' using 100 µL distilled water mixed with 1mL Bradford reagent. The absorbance at 595 nm, of unknown protein samples was used to calculate the protein concentration via the equation of a previously determined standard curve. The preparation of the standard curve is described in appendix 1.

2. 10 Seed Storage Protein Development Study

Two crops of thirty large third generation *Sandersonia* tubers were planted on 1/6/00 and 21/9/00 respectively. The crops were planted in pots as described above. The crops were watered depending on season. From the date of planting until first flower emergence the first crop (a winter crop) was watered 150ml twice weekly and then 200ml twice weekly until tuber excavation (post flower harvest). The second crop (a spring/summer crop) was watered 200ml twice weekly from planting until first flower emergence and then 250ml twice weekly until mid flower harvest. Finally, until tuber excavation the crop was watered by hand until excess water appeared below the pot.

Both crops produced adult plants bearing flowers 1.5-2 months after planting.

Flowers were determined to be mature and fully opened when the green tips of the hanging petals curled outwards changing to a yellow/cream and eventually golden orange colour matching the rest of the petal. The day the petal tips rolled outwards with the aforementioned colour change was recorded as the flowering day (anthesis). On the day of flowering each flower was labelled with a small paper label affixed with string, noting its parent plant number and individual flower number. The second day after flowering was recorded as the pollination day. For the next two days (for three days in total) flowers were pollinated by hand using a medical cotton tip. Cross pollination occurred as randomly as possible, but was dependent on the number of other flowers bearing pollen at that time. Pollination occurred over a two-week period for the first crop and a three-week period for the second crop.

In the first crop, 220 flowers were harvested following a planned schedule. Flowers were harvested 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 days after pollination. Twenty flowers were harvested at each of the eleven maturity groups.

Whole flowers were harvested from the adult plant and taken to the laboratory in a plastic container for dissection. The flowers were dissected by scalpel on a

petri dish lined with moist filter paper. The seeds were removed by forceps and stored in labelled eppendorf tubes at -80°C until analysis.

In the second crop, 275 flowers were harvested following a similar scheme. Twenty five flowers were harvested at each of the eleven maturity groups and handled as mentioned above. The seeds of the two crops were analysed via SDS PAGE.

2. 11 Tuber Storage Protein Development Study

Seven large third generation *Sandersonia* mother tubers were planted on 21/9/00 in pots. The tubers were planted in conditions as described above. The tubers were watered by the same schedule as described for the second crop in the 'Seed Storage Protein Development Study'.

One mother tuber was harvested each week, for six weeks. The tubers were harvested, following the developmental sequence from least mature- consisting of only a forked tuber, to most mature- consisting of a forked tuber plus one shoot and accompanying roots. Each tuber was excavated by hand and taken to the laboratory in a sealed plastic container.

Using a scalpel and forceps, the tubers were sampled on a dry petri dish. The forked tubers were sampled in six locations. Two pieces of tuber tissue, each approximately 5mm, were dissected from, the centre of the fork, the mid point between the fork and the tip, and the tips of the tubers. The tuber tissue samples were placed in labelled and punctured eppendorf tubes and rapidly frozen in liquid nitrogen. The samples were stored at -80°C until analysis via SDS PAGE.

2. 12 Seed Dry Weight Analysis

A total of six dry weight analyses were carried out. Seeds representing each of the two crops were used, with three replicates for each. In each replicate, 20 seeds of each developmental age were dried. Eleven developmental ages were represented (10-60 DAP).

Due to logistical reasons, the fresh weights of seeds were not taken directly after harvest. Instead, seeds stored at -80°C were defrosted for three hours then weighed. Within that time the seeds were placed in aluminium foil parcels. The parcels of seeds were dried in a bench top laboratory oven at 60°C for seven days. The seeds were considered dry when two consecutive weights appeared the same.

2. 13 Light Microscopy and TEM

2. 13. 1 Seed Sample Fixation and Embedding

Seeds aged 11, 20, 29, 40, 51 and 62 DAP were prepared for light microscopy and transmission electron microscopy after a series of steps, as described below.

1. Selected, freshly harvested seeds were prepared for fixation by partial dissection. Cuts were made on either side of the endosperm along the longitudinal axis of the seed, to allow complete fixative infiltration.
2. After dissection, seeds were immediately placed in glass vials of infiltration buffer (see appendix 1) containing 3% glutaraldehyde (v/v). Seeds were fixed in this solution by vacuum infiltration overnight, with the vial lids removed.
3. Seed containing vials were removed from vacuum and the vial lids immediately reattached. After this seeds were subjected to three, ten minute infiltration buffer (minus glutaraldehyde) rinses administered by glass pipette.
4. Fixed seeds were then immersed in 1 % osmium (v/v) solution for three hours.
5. The seeds were then rinsed once for ten minutes in infiltration buffer (minus glutaraldehyde), then subjected to a series of four 10 minute dehydration rinses in

acetone. The acetone rinses increased stepwise in concentration, 20%-80% acetone (v/v).

6. Seeds were dehydrated by three, 10 minute rinses with 100% acetone, in the same sealed glass vials.

7. Seeds were then immersed in a one third Spurr's resin/ two thirds 100% acetone mix overnight. During this time, vials were placed in a continuous, circular rotating agitator.

8. Seeds were immersed in a two thirds Spurr's resin/ one third 100% acetone mix for three hours. The same rotary agitator was used.

9. Seeds were then transferred to small plastic caps and immersed in 100% Spurr's resin and baked for eight hours at 65°C in a bench top laboratory oven.

After baking, solid resin disks were removed from their caps and stored at room temperature.

2. 13. 2 Preparation for Light Microscopy

An individual seed in each resin disk was identified for TEM viewing. The seeds were cut from their disks by saw and affixed, in correct orientation, to a resin stud with araldite (see appendix 1). The resin seed block was clamped into an ultramicrotome via the attached resin stud. Surplus resin was trimmed from each resin block by razor blade to create a flat sectioning surface. The flat surface was aligned with the ultramicrotome's glass blade, by which the block was trimmed until the specimen could be completely visualised in each section. 2.5 µm optical sections were cut for light microscope observation. These sections were flame-fixed to slides and stained for 2 minutes with a 50:50 mixture of azur blue and methylene blue. The slides were rinsed with water and a cover slip affixed with DPX

mounting medium. The slides were observed under light microscope and suitable sites for TEM were chosen.

2. 13. 3 Preparation for TEM

The resin blocks were trimmed further by razor blade until only fixed seed tissue was exposed and all surrounding resin was removed. All fixed seed tissue immediately around the TEM intended site was removed by razor blade. Seed tissue was sectioned for TEM use by an automated LBK Bromma 2128 ultramicrotome using a diamond blade. 100 nm sections of seed tissue were stained with uranyl acetate and lead citrate before being mounted on grids with and without support film. Specimens were then viewed under TEM.

CHAPTER 3

RESULTS

3.1 Initial Crop Growth

Twelve tubers were purchased from a local garden centre in late November 2000, a time of year considered 'late' for planting outdoors. At the time of possession the tubers had already sprouted shoots and roots in their packaging.

The tubers were planted in a vermiculite (lower layer)/potting mix (upper layer) media and placed in an incubator room (the same room used to house crop 0). Three weeks after planting, the established shoots withered and no new shoots appeared. The tubers were excavated and chilled (at 4°C) in plastic bags for 11 weeks (Clark, 1997). After the dormancy-breaking chilling period had elapsed, the tubers were incubated at 23°C for 7 days. This period was intended to induce shoot sprouting, though there was no noticeable effect on the tuber tips after treatment. The tubers (known as crop 0) were replanted in the same media, but with media layers reversed. Four weeks later only 2 of 12 tubers successfully sprouted a shoot each. After another 4 weeks only 3 flowers were produced between the two plants. The remaining ten tubers were found to have decomposed over the two month period, since planting. The 3 mature flowers were successfully cross-pollinated and bore seedpods containing seeds.

Crop 1 consisted of 30 tubers purchased from a commercial grower/exporter. All tubers planted in crop 1, produced shoots 4-6 weeks after planting, though 2 tubers' shoots did not reach maturity. Of the 30 tubers, 28 bore flowers. Approximately 250 flowers in total were produced across the crop, with plants' individual contributions ranging from 1-20 flowers. These flowers were randomly cross-pollinated and produced as many seedpods bearing seeds. The majority of adult plants, at the post-flowering stage grew to twice the height of those in crop 0. A second wave of smaller flowers was produced after the main flowers had wilted.

The production of these flowers was separated from the main body of flowers by approximately two weeks and coincided with the plants reaching their maximum heights. Four months after planting, all remaining mature (>60 DAP) flowers were harvested and the tubers excavated and stored at 4°C.

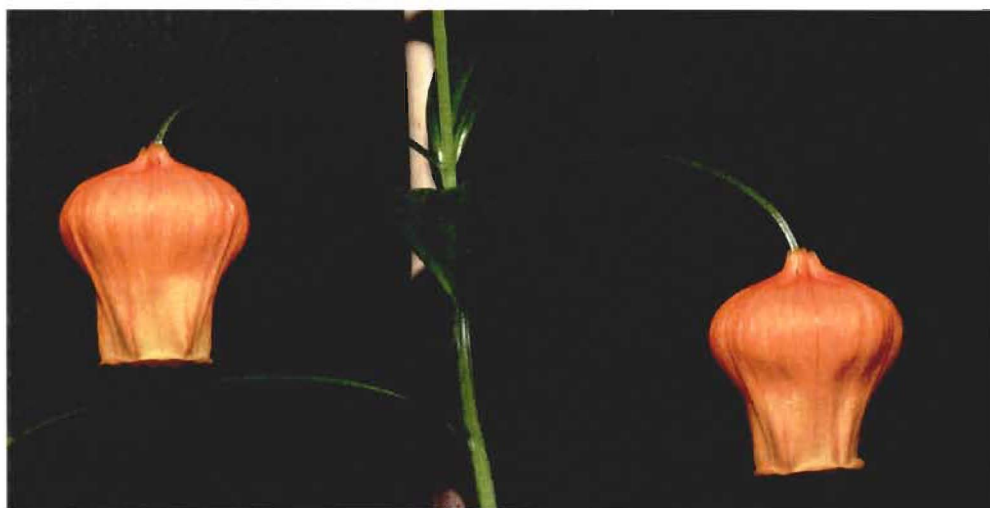
Crop 2 consisted of another 30 tubers purchased from the same supplier, plus seven additional tubers (to be used in the tuber protein development study). The tubers were the same size as used in crop 1, only varying in planting date and season of planting. Of the 30 tubers, 29 sprouted shoots within 4-6 weeks of planting and those same individuals produced flowers. Approximately 350 flowers were produced across the crop, with individual plants' contributions ranging from 4-37 flowers. As with crop 1, all flowers were randomly cross-pollinated to produce seed pods bearing seeds. Also, the majority of adults grew to the same height as those in crop 1 and produced a second wave of smaller flowers. Four months after planting, the remaining mature flowers produced by crop 2 were harvested and the parent tubers excavated and stored at 4°C.

Both crops 1 and 2 had a small number of extremely vigorous tubers each producing a second smaller shoot. These shoots originated from the dormant 'arm' of the forked tuber. Flowers produced by these secondary shoots were included in the total flower number for that tuber and were considered under the same conditions as the main shoots' flowers.

Crop 3 consisted of the tubers of crop 1, which had been in storage at 4°C for 15 weeks. The tubers were planted in the same media as crops 1 and 2. Of the 30 tubers, 19 sprouted shoots, 4-6 weeks after planting and all of those produced flowers. The flowers of crop 3 were smaller than the other crops'.

A

2mm

B

3cm

Plate 1 A and B. A, A germinated Sandersonia seed, B, mature Sandersonia flowers.

3. 2 Extraction of Seed Proteins

The efficiency of four buffers of different composition, for the extraction of *Sandersonia aurantiaca* seed protein, was compared after SDS PAGE and Coomassie blue gel staining. Mature seeds, aged greater than 60 DAP were examined. Plates 2, 3 and 4 show the extraction efficiencies of phosphate buffer, sample buffer, crystalloid protein extraction (CPE) buffer and water respectively.

Plate 2 shows a comparison of phosphate and sample buffers. Equal quantities of fresh weight seed meal (40 mg) were ground and extracted individually with the two buffers (1 mL each), with three replicates each. Both buffers were able to extract the same 6 major protein bands. The sample buffer extracted gel bands appeared more intense than the phosphate buffer bands but the protein bands of both buffers were considered to have the same molecular masses.

Plate 3 shows a SDS PAGE comparison of phosphate buffer alone, phosphate buffer initially followed by a pellet reextraction with CPE buffer² (1 mL) and sample buffer initially followed by a pellet reextraction with CPE buffer (1 mL), all with and without mercaptoethanol. The gel image (plate 3) shows that with each of the four treatments, the same set of protein bands are extracted and no new protein bands are present when compared with lane 1. Also the gel shows that the reductive effect of mercaptoethanol did not result in additional protein bands. The 6 major seed protein bands are individually visible in plate 3.

Plate 4 shows a SDS PAGE comparison of the extraction efficiencies of phosphate buffer and water. The gel image shows that traces of protein were extracted with water, though the six major protein bands were not extracted with water.

² CPE (Crystalloid Protein Extraction) buffer is a 50:50 mixture of phosphate buffer and CPE buffer (see Appendix 1).

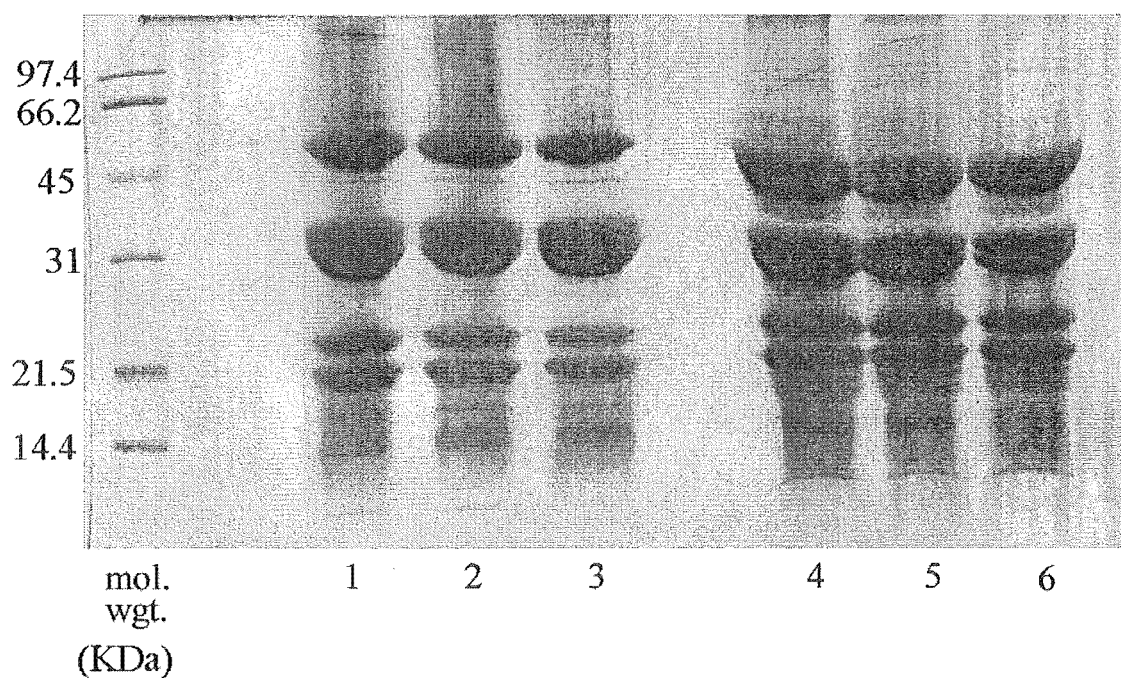


Plate 2. SDS PAGE comparison of phosphate and 'sample' buffer extracted mature seed protein. Lanes 1-3 show phosphate buffer extracted protein. Lanes 4-6 show sample buffer extracted protein. Ten microlitres of protein were loaded per lane. Five microlitres of Bio-Rad molecular weight markers were loaded. A 12% acrylamide gel was used.

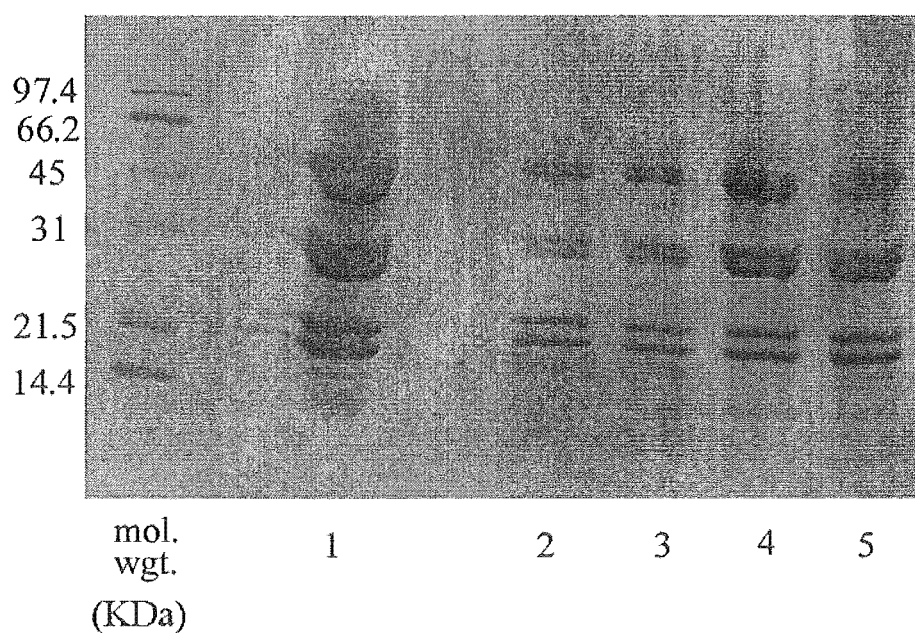


Plate 3. SDS PAGE comparison of mature seed protein extracted by phosphate buffer and CPE buffer. Lane 1; phosphate buffer extracted protein, lane 2; sample buffer followed by CPE buffer +mercaptoethanol, lane 3; sample buffer followed by CPE buffer -mercaptoethanol, lane 4; phosphate buffer followed by CPE buffer +mercaptoethanol, lane 5; phosphate buffer followed by CPE buffer -mercaptoethanol. Ten microlitres of protein were loaded per lane. Five microlitres of Bio-Rad molecular weight markers were loaded. A 12% acrylamide gel was used.

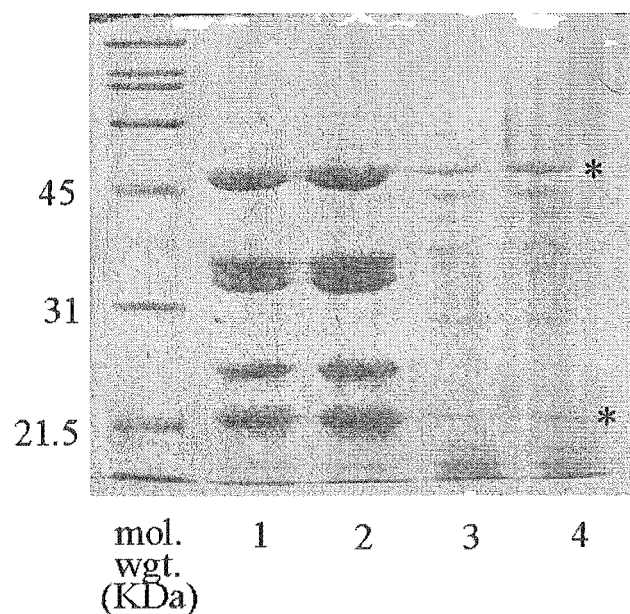


Plate 4. SDS PAGE comparison of phosphate buffer and water extraction of mature seed protein. Lanes 1 and 2; phosphate buffer extracted protein, lanes 3 and 4; water extracted protein. Ten microlitres of protein were loaded per lane. Five microlitres of Bio-Rad molecular weight markers were loaded. A 12 % acrylamide gel was used. The asterix indicate water soluble proteins that may correspond to salt soluble proteins of the same size.

3. 3 Extraction of Tuber Proteins

The efficiency of tuber protein extraction was compared between four different types of extraction buffers. The extraction efficiency was compared directly through SDS PAGE protein banding. The buffers used were the same as those used in section 3. 2. Dormant, pre-flowering stage (1st generation daughter) tubers were examined (from crop 0 tuber shipment).

Plate 5 shows a SDS PAGE comparison of the tuber protein extraction efficiencies of phosphate buffer, sample buffer, CPE buffer and water. Gel lanes were standardised by using tuber tissue samples of the same fresh weight. Lanes 1 and 2 show water extracted tuber protein, lanes 3 and 4 show phosphate buffer extracted tuber protein, lanes 5 and 6 show sample buffer extracted tuber protein and lanes 7 and 8 show CPE buffer extracted tuber protein. In each instance 1 mL buffer was used to extract protein from \approx 0.30 g tuber tissue samples.

The gel image shows all four buffers were able to extract four major protein bands. Also, with slightly varying success, all four buffers were able to extract minor, heavier protein bands. Interestingly, lanes 1 and 2 show that the major tuber proteins are water soluble.

Plate 6 shows a SDS PAGE comparison of the tuber and seed proteins extracted with phosphate buffer. Lane 1 shows the protein extracted from 39 mg seed meal with phosphate buffer (1 mL). Lane 2 shows the protein extracted from approximately 0.5 g tuber tissue with phosphate buffer (1 mL).

The gel image shows that mature seeds and dormant (daughter) tubers have their own sets of major proteins, which differ in molecular weights, demonstrated by the different protein banding migrations. The major seed proteins appear as 6 mid to heavy weight bands, whereas the major tuber proteins appear as 4 increasingly lightweight bands. The gel also shows that none of the minor, heavier tuber protein bands have the same molecular weight as any seed protein band.

Table 1 shows a comparison of the seed and tuber major protein molecular masses.

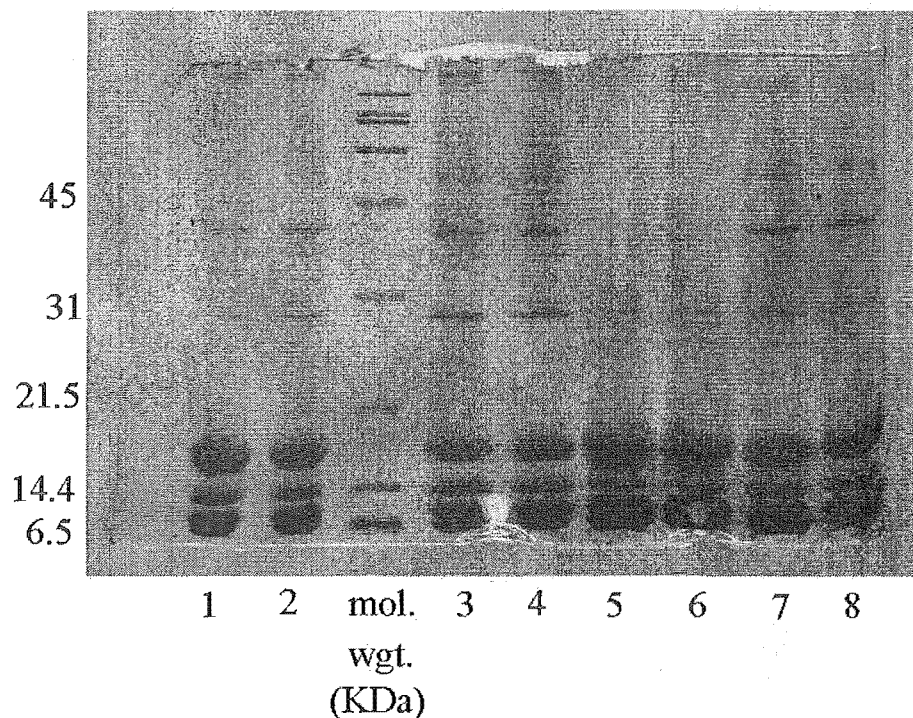


Plate 5. SDS PAGE comparison of post-flowering daughter tuber protein extracted with water, phosphate, sample and CPE buffers. Lanes 1 and 2; water extracted protein, lanes 3 and 4; phosphate buffer extracted protein, lanes 5 and 6; sample buffer extracted protein, lanes 7 and 8; CPE buffer extracted protein. Ten microlitres of protein were loaded per lane, 5.5 microlitres of Bio-Rad molecular weight markers were loaded. A 12 % acrylamide gel was used.

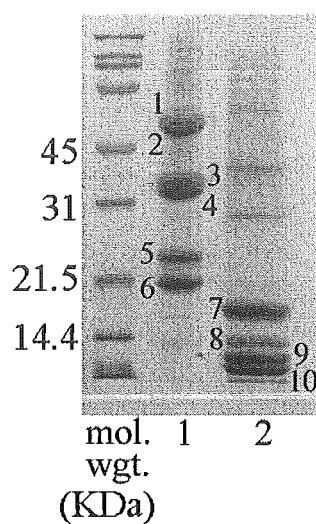


Plate 6. SDS PAGE comparison of phosphate buffer extracted mature seed and post-flowering daughter tuber protein. Lane 1; phosphate buffer extracted seed protein, lane 2; phosphate buffer extracted tuber protein. Ten microlitres of protein were loaded per lane. Five microlitres of Bio-Rad molecular weight markers were loaded. A 12 % acrylamide gel was used. Gel band numbers correspond to table 1.

Seed Bands	Molecular Mass (KDa)
1	54.9
2	51.9
3	36.3
4	32.5
5	20.9
6	17.2
Tuber Bands	
7	16.8
8	13.7
9	12.2
10	11.5

Table 1. Comparison of *Sandersonia* seed and tuber major proteins and their approximate molecular masses. Band numbers correspond to plate 6.

3. 4 Seed Storage Protein: A Development Study

Two crops (1 and 2) of tubers were planted and their seeds harvested at eleven intervals ranging from ten to sixty days (seed maturity) after pollination. The growth and biochemical change of these seeds was monitored.

3. 4. 1 Seed Development

The development of seed growth was monitored through four characteristics: fresh weight, dry weight, water content and total protein content. The fresh weight of immature seeds of both crops, was found to increase dramatically from 10- approximately 30 DAP (figure 1). From 30 to 60 DAP the rate of increase was less extreme. Figure 3 shows the water content of seeds increasing sharply until 25 to 30 DAP, where it stays at a roughly steady level. After 60 DAP the seeds began to visually desiccate (data not shown) and were considered mature. The dry weight of seeds steadily increased to a maximum at 55 DAP (figure 2). This coincided with the increase in total seed protein (figure 4), which increased steadily over the range of sampling ages.

3. 4. 2 Variance between Crops

The two crops used for study were planted at slightly different times of year. The glasshouse temperatures experienced by crop 2 were slightly higher than those experienced by crop 1. In the seed fresh weight analysis (figure 1) the trends are the same among the different plantings, but the average fresh weight of crop 2 seeds were higher than those of the first crop within the first 25 days of development. After 30 DAP the average fresh weight of each crops seeds stayed roughly the same.

In the seed dry weight analysis (figure 2) the crop trends are similar, but the second crops dry weights are consistently higher than the first crops. In the seed water content analysis (figure 3) the second crop attained a higher water level than

the first crop within the first 25 DAP. Over the remaining developmental dates the two crops' water content remained roughly constant. In the total seed protein analysis (figure 4), the trends of both crops are similar though crop 2 seeds increased their total protein markedly during the period 35-45 DAP.

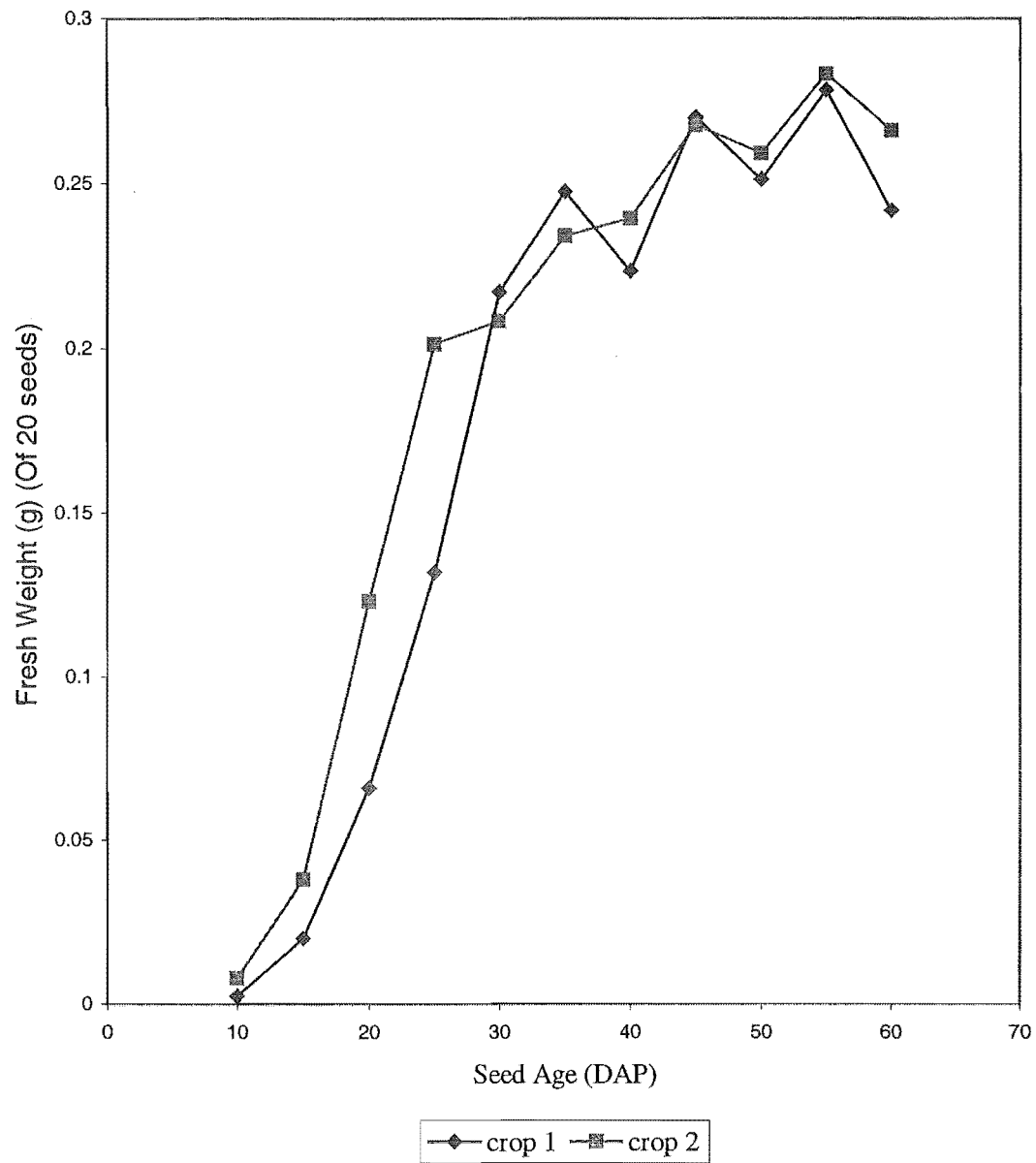


Figure 1. Inter-crop comparison of fresh weight (g) of 20 seeds, harvested at eleven developmental ages (DAP). Each data point represents the average of 3 replicates of 20 seeds.

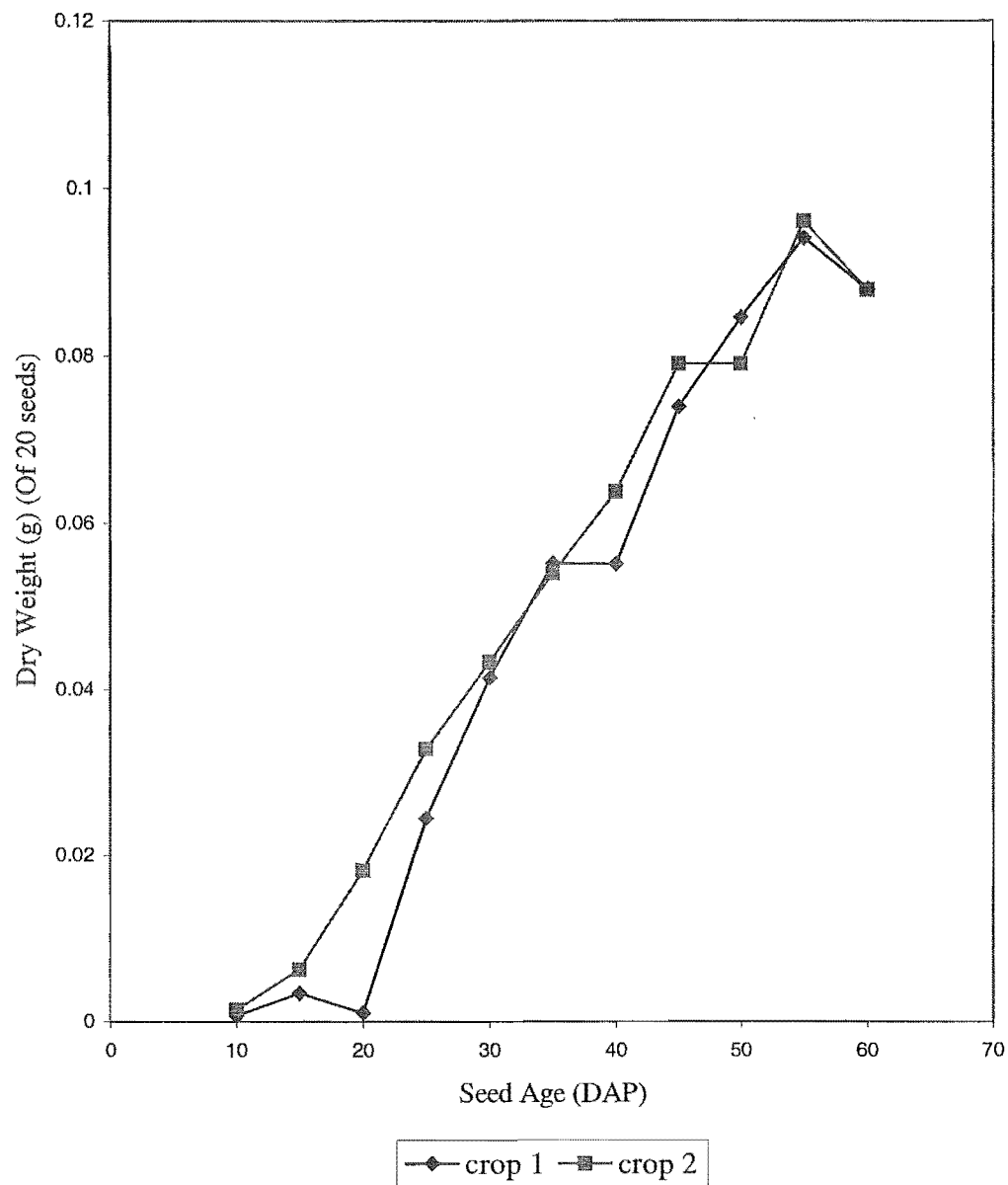


Figure 2. Inter-crop comparison of the dry weight (g) of 20 seeds, harvested at eleven developmental ages (DAP). Each data point represents the average of 3 replicates of 20 seeds.

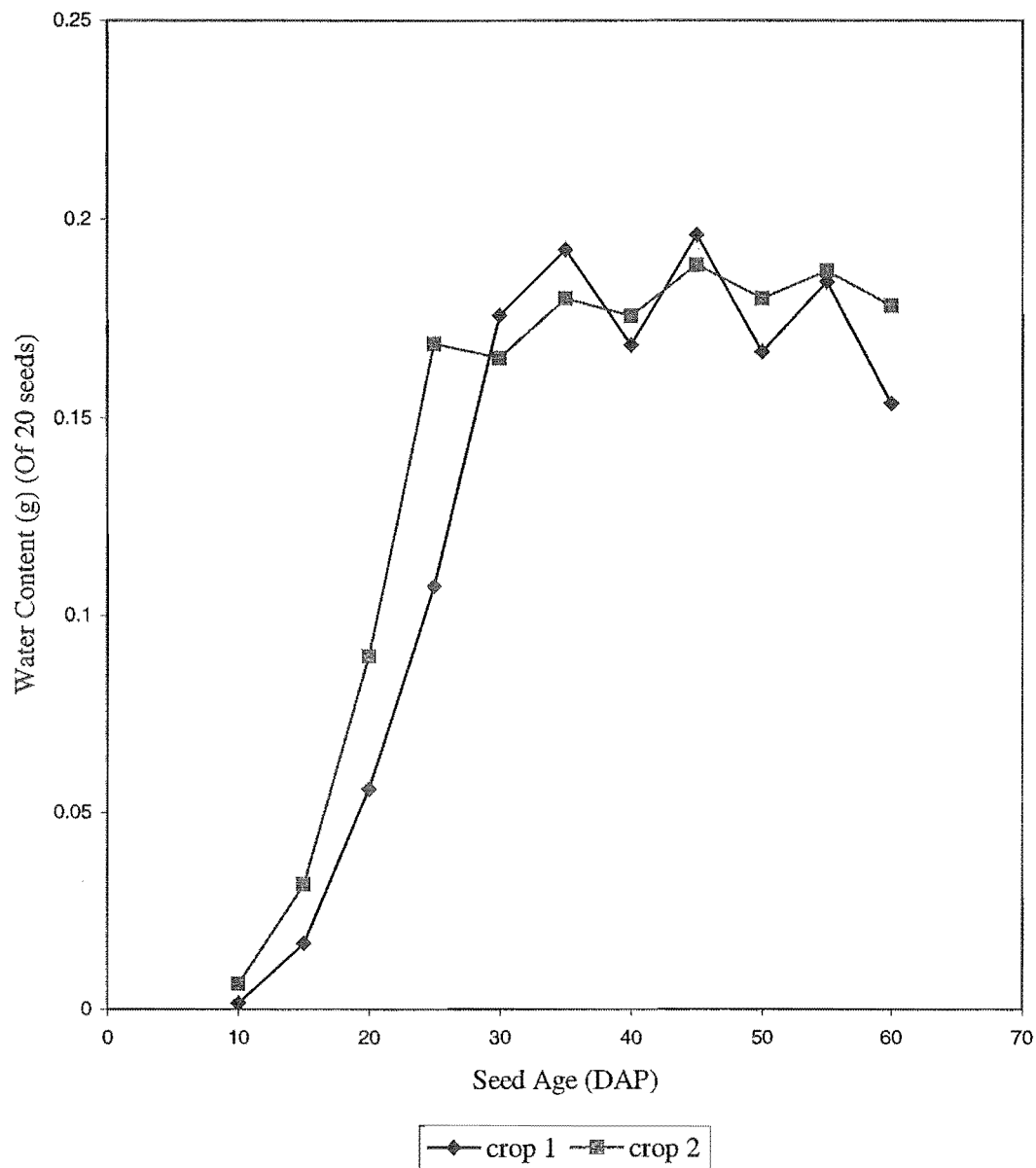


Figure 3. Inter-crop comparison of the water content (g) of 20 seeds, harvested at eleven developmental ages (DAP). Each data point represents the average of 3 replicates of 20 seeds.

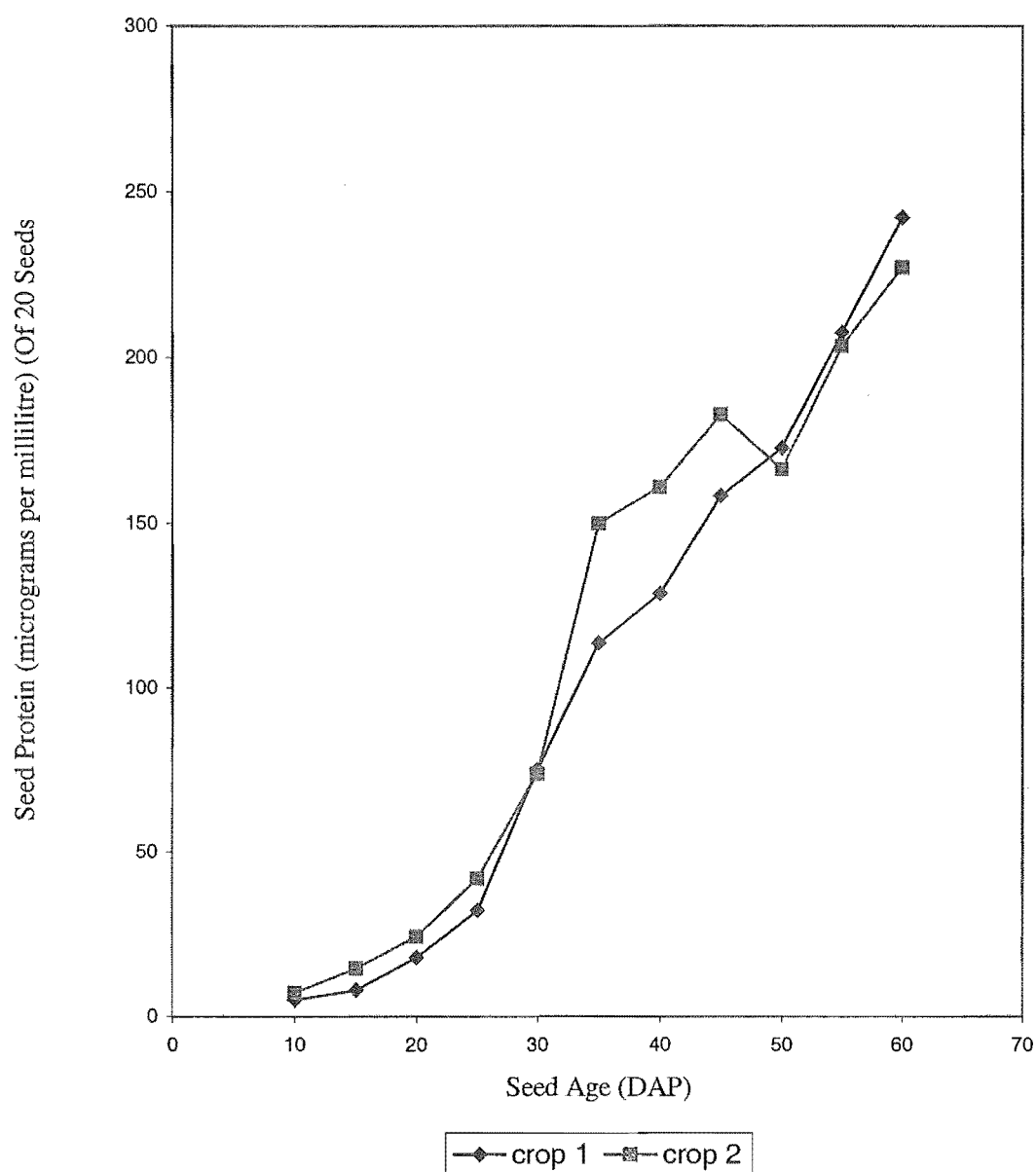


Figure 4. Inter-crop comparison of the total extractable seed protein (micrograms per millilitre) of 20 seeds, harvested at eleven developmental ages (DAP). Each data point represents the average of 3 replicates of 20 seeds.

3. 4. 3 SDS PAGE

The formation of the major seed proteins in developing seeds was monitored at intervals by SDS PAGE. Plate 7 shows a SDS PAGE comparison of seed protein (from crop 2 seeds) extracted with phosphate buffer. At each developmental age, protein was extracted from 20 seeds (of the same flower) with the exception of the 10 DAP sample which had to be prepared by combining seeds from three flowers, to obtain a sufficiently concentrated protein extract. All lanes had equivalent protein loading, standardised using the Bradford assay. The gel image shows that protein is present 10 and 20 DAP though the main storage bands are not present. Lane 3 shows the main storage proteins have formed by 30 DAP. The main storage protein bands increased in intensity to a maximum, around 50 DAP.

Plate 8 shows a SDS PAGE comparison of seed protein (from crop 1 seeds) extracted with phosphate buffer. At each developmental age, protein was extracted from 20 seeds, with all lanes having equivalent protein loading. The gel image confirms that the main storage proteins are formed 30 DAP. At 25 DAP only minor bands are present. By 35 DAP the major bands have formed and these increase in intensity by 45 DAP.

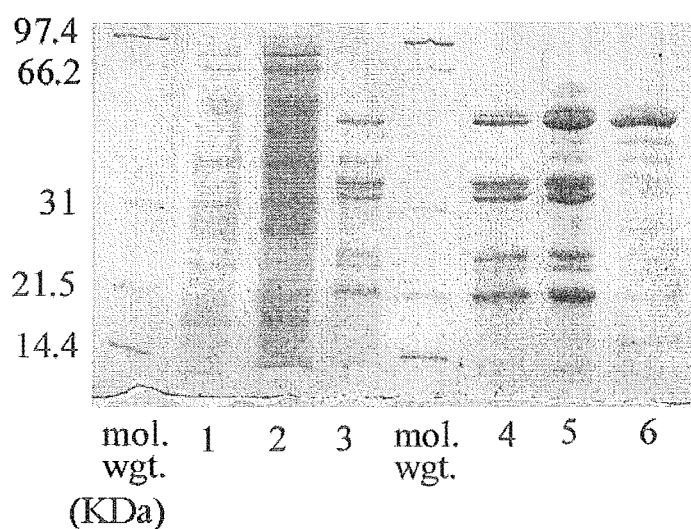


Plate 7. SDS PAGE comparison of phosphate buffer extracted seed protein, from seeds harvested 10-60 DAP. Lane 1; protein extracted from 10 DAP seeds, lane 2; protein extracted from 20 DAP seeds, lane 3; protein extracted from 30 DAP seeds, lane 4; protein extracted from 40 DAP seeds, lane 5; protein extracted from 50 DAP seeds, lane 6; protein extracted from 60 DAP seeds. Ten microlitres of protein were loaded per lane. Lanes were standardised by loading 70-75 micrograms per millilitre. Six microlitres of Bio-Rad molecular weight markers were loaded. A 12 % acrylamide gel was used.

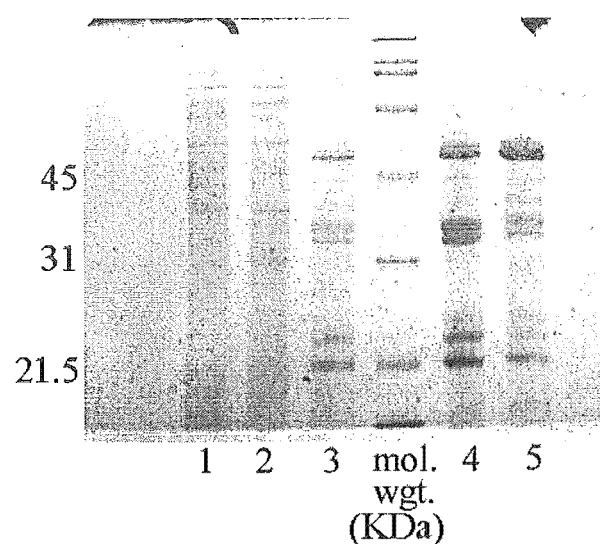


Plate 8. SDS PAGE comparison of phosphate buffer extracted seed protein, from seeds harvested 15-55 DAP. Lane 1; seed protein 15 DAP, lane 2; seed protein 25 DAP, lane 3; seed protein 35 DAP, lane 4; seed protein 45 DAP, lane 5; seed protein 55 DAP. Ten microlitres of protein were loaded per lane. Lanes were standardised at 60-70 micrograms per millilitre. Five microlitres of Bio-Rad molecular weight markers were loaded. A 12 % acrylamide gel was used.

3. 5 Tuber Storage Protein Development Study

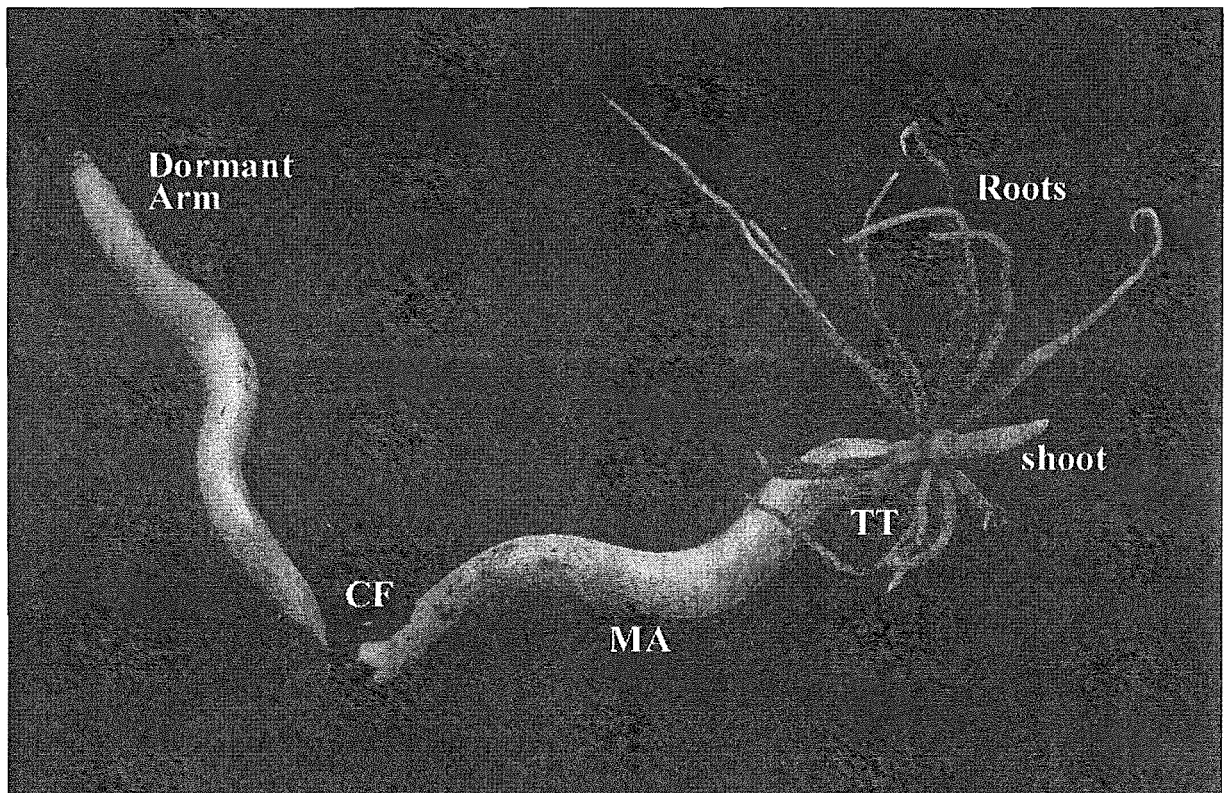
3. 5. 1 SDS PAGE

Seven large tubers were planted concurrently, and one tuber harvested for sampling each week, for six weeks. The tubers were sampled in three locations: ‘tuber tip’, ‘mid arm’ and ‘centre of fork’. Plate 9 shows a tuber harvested 3 weeks after planting and the tuber locations of tissue sampling. The tissue samples were analysed by SDS PAGE, in an effort to observe any changes in tuber storage protein that might occur during shoot formation. Gel protein loading was standardised by the Bradford assay.

Plate 10 shows a SDS PAGE comparison of tuber tissue samples extracted with phosphate buffer. Lanes 1-7 show tuber tissue samples originating from the ‘centre of fork’ location, each representing a different maturity level. The gel image shows that all four tuber storage protein bands are present, at the same intensity for the first six weeks of new season tuber growth.

Plate 11 shows a SDS PAGE comparison of tuber tissue samples extracted with phosphate buffer. Lanes 1-6 show tuber tissue samples originating from the ‘tuber tip’ location, each representing a different maturity level. The gel image shows that all four tuber storage protein bands are present, at the same intensity for the first six weeks of new season tuber growth.

Plate 12 shows a SDS PAGE comparison of tuber tissue samples extracted with phosphate buffer. Lanes 1-7 show tuber tissue samples originating from the ‘mid arm’ location, each representing a different maturity level. The gel image shows that all four tuber storage protein bands are present, at the same intensity for the first six weeks of new season growth.



25mm

Plate 9. Sandersonia mother tuber harvested 21 days after planting. The new season shoot and roots are visible. **T**; 'Tuber Tip' sample location, **MA**; 'Mid Arm' sample location, **CF**; 'Centre of Fork' sample location.

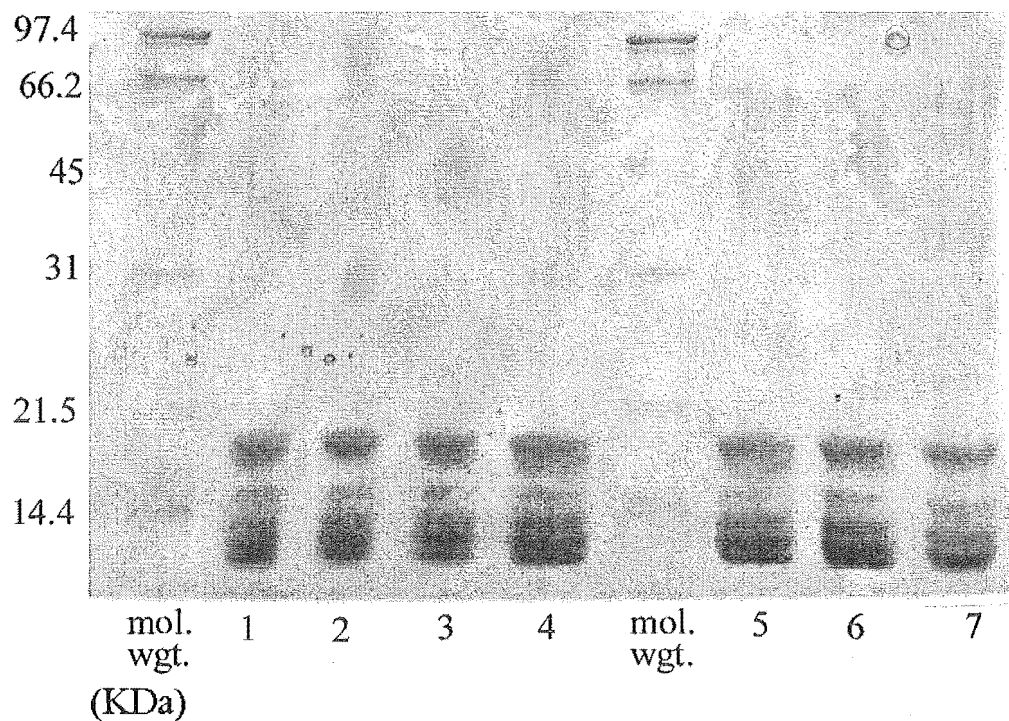


Plate 10. SDS PAGE comparison of phosphate buffer extracted tuber protein, from tubers harvested 0-6 weeks after new season planting, sampled at the 'centre of fork' location. Lane 1; tuber protein of an unplanted nondormant tuber, lane 2; tuber protein of a 1 week old harvested tuber, lane 3; tuber protein of a 2 week old harvested tuber, lane 4; tuber protein of a 3 week old harvested tuber, lane 5; tuber protein of a 4 week old harvested tuber, lane 6; tuber protein of a 5 week old harvested tuber, lane 7; tuber protein of a 6 week old harvested tuber. Twelve microlitres of protein were loaded per lane. Lanes were standardised by loading 32-42 micrograms per millilitre. Six microlitres of Bio-Rad molecular weight markers were loaded. A 12 % acrylamide gel was used.

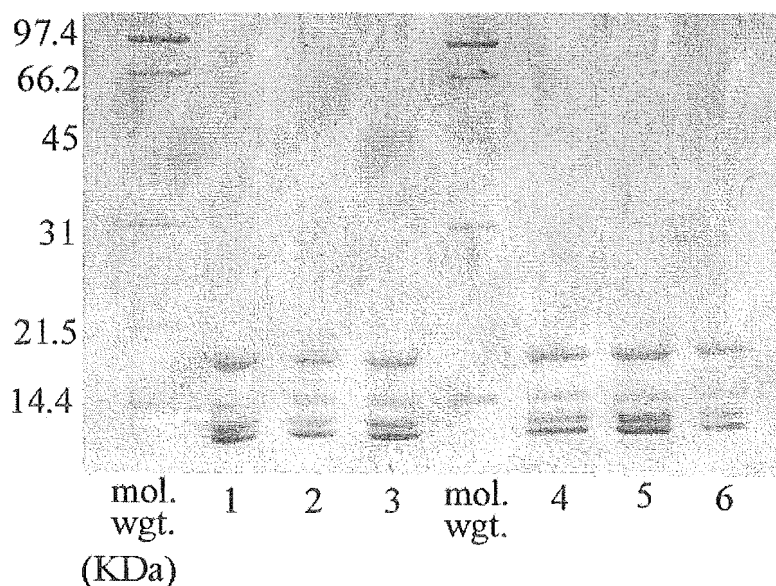


Plate 11. SDS PAGE comparison of phosphate buffer extracted tuber protein from tubers harvested 0-6 weeks after new season planting, sampled at the '**tuber tip**' location. Lane 1; tuber protein from an unplanted, nondormant tuber, lane 2; tuber protein from a 1 week old harvested tuber, lane 3; tuber protein from a 2 week old harvested tuber, lane 4; tuber protein from a 3 week old harvested tuber, lane 5; tuber protein from a 4 week old harvested tuber, lane 6; tuber protein from a 6 week old harvested tuber. Twelve microlitres of protein were loaded per lane. Lanes were standardised by loading 40-42 micrograms per millilitre. Six microlitres of Bio-Rad molecular weight markers were loaded. A 12 % acrylamide gel was used.

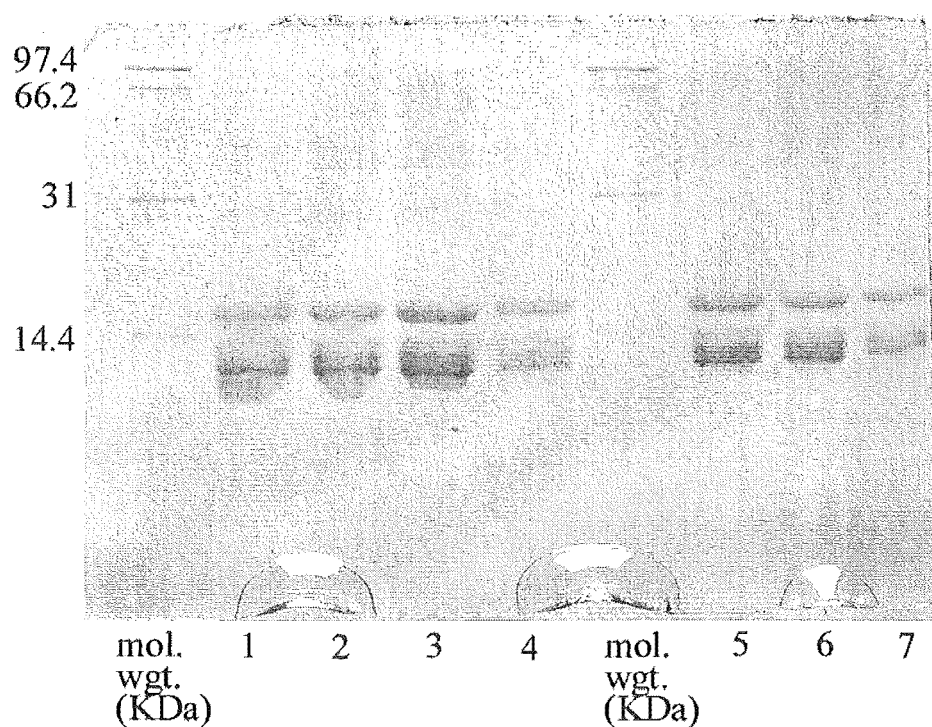


Plate 12. SDS PAGE comparison of phosphate buffer extracted tuber protein from tubers harvested 0-6 weeks after new season planting, tissue sampled from the 'mid arm' location. Lane 1; protein extracted from a nondormant, unplanted tuber, lane 2; protein extracted from a 1 week old harvested tuber, lane 3; protein extracted from a 2 week old harvested tuber, lane 4; protein extracted from a 3 week old harvested tuber, lane 5; protein extracted from a 4 week old harvested tuber, lane 6; protein extracted from a 5 week old harvested tuber, lane 7; protein extracted from a 6 week old harvested tuber. Ten microlitres of protein were loaded per lane. Lanes were standardised by loading 45-52 micrograms per millilitre protein. Six microlitres of Bio-Rad molecular weight markers were loaded. A 15 % acrylamide gel was used.

As no difference was noted in protein banding (regarding band location or intensity) during the first six weeks of new season tuber growth, an extension of the experiment was conducted. Three tubers from crop 3 were harvested individually at three further developmental stages. The mother tubers were again sampled in distinct locations (tip, centre of fork and mid arm). The three maturity stages were:

1. An adult pre-flowering stage plant bearing 8 immature flower buds,
2. A 64 cm adult mid-flowering plant bearing 7 mature flowers (orange) and 9 immature flowers (green),
3. A 95 cm adult post-flowering stage plant bearing 34 flowers, one third of those pollinated, bearing immature seed pods.

Plate 13 shows a SDS PAGE comparison of tuber tissue samples extracted with phosphate buffer. Lanes 1-9 show tuber tissue representing the final three developmental stages sampled and the three mother tuber sample locations. The lanes were standardised by loading protein samples extracted from tissue samples of similar fresh weight. The gel image shows that at the first two sampling stages (1 and 2 above), represented in lanes 1-6, the tuber storage proteins are still present at a high intensity. Gel lanes 7-9 represent the mature most sampling age. They show the main tuber storage proteins are no longer present at high intensity and certain specific lower molecular weight bands are missing altogether. This result was found at each of the three sampling locations on the mother tuber (represented by a lack of storage protein banding in lanes 7-9).

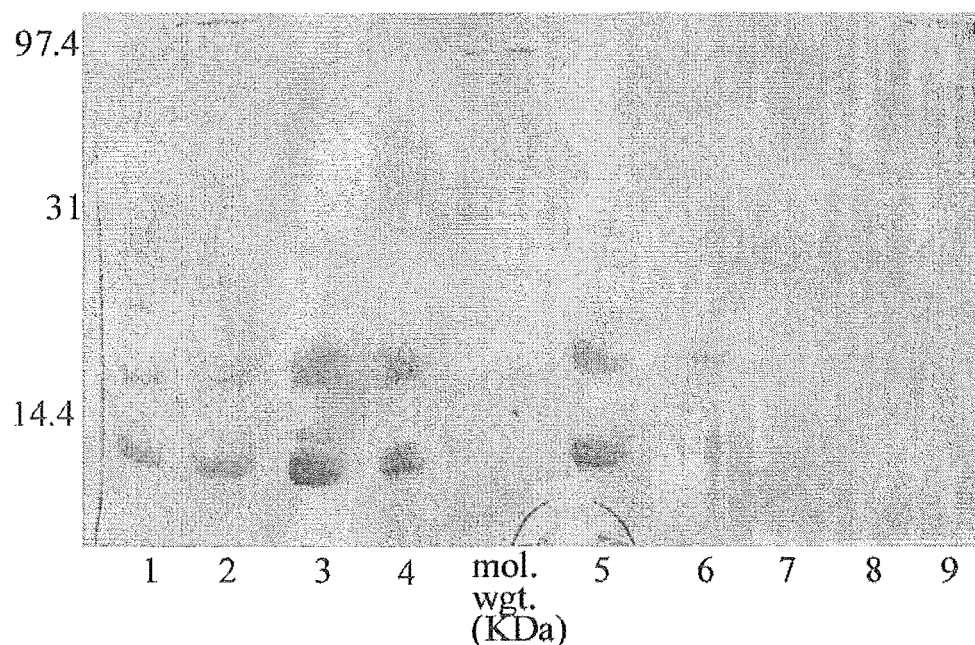


Plate 13. SDS PAGE comparison of phosphate buffer extracted tuber protein, from mother tubers harvested at successively advanced lifecycle stages, at 3 sampling locations on the tuber. Lane 1; stage 1 tuber protein sampled from 'tip' location, lane 2; stage 1 tuber protein sampled from 'mid arm' location, lane 3; stage 1 tuber protein sampled from 'centre of fork' location, lane 4; stage 2 tuber protein sampled from 'tip' location, lane 5; stage 2 tuber protein sampled from 'mid arm' location, lane 6; stage 2 tuber protein sampled from 'centre of fork' location, lane 7; stage 3 tuber protein sampled from 'tip' location, lane 8; stage 3 tuber protein sampled from 'mid arm' location, lane 9; stage 3 tuber protein sampled from 'centre of fork' location. Ten microlitres of extracted protein were loaded per lane. Lanes were standardised by loading 39-49 micrograms per millilitre protein. Six microlitres of Bio-Rad molecular weight markers were loaded. A 12 % acrylamide gel was used.

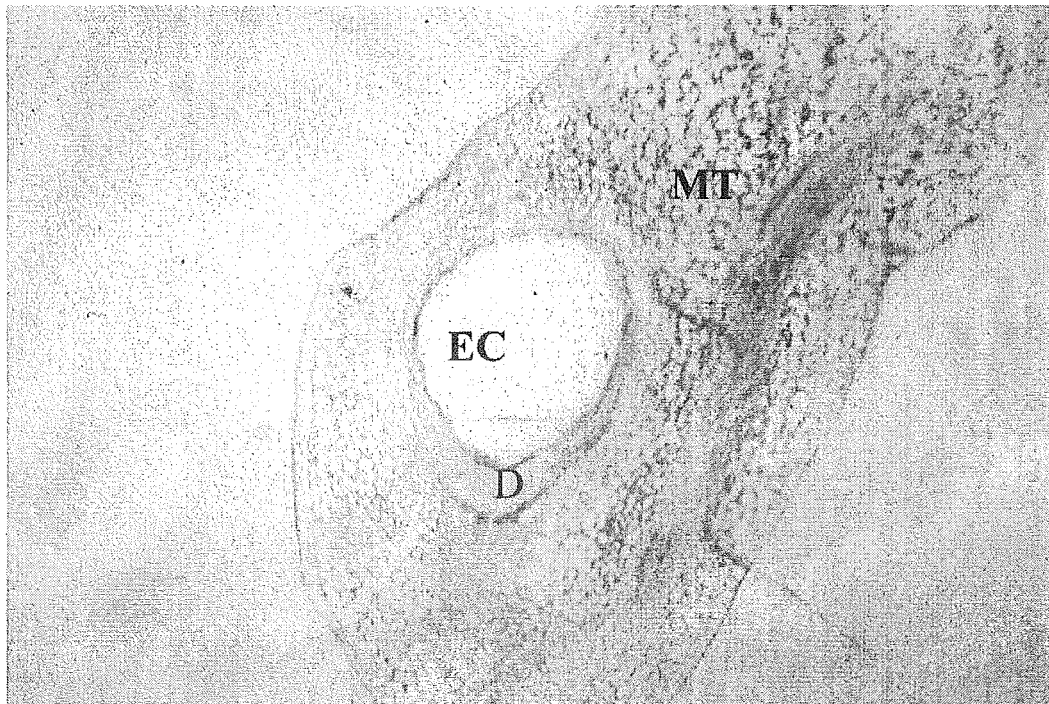
3. 6 Seed Development: Microscopic Observations

Light microscopy and transmission electron microscopy was carried out on fixed (3% glutaraldehyde/osmium), microtomed seed tissue samples. Seeds representing a range of DAP were studied, in order to better understand seed development.

3. 6. 1 Light Microscopy

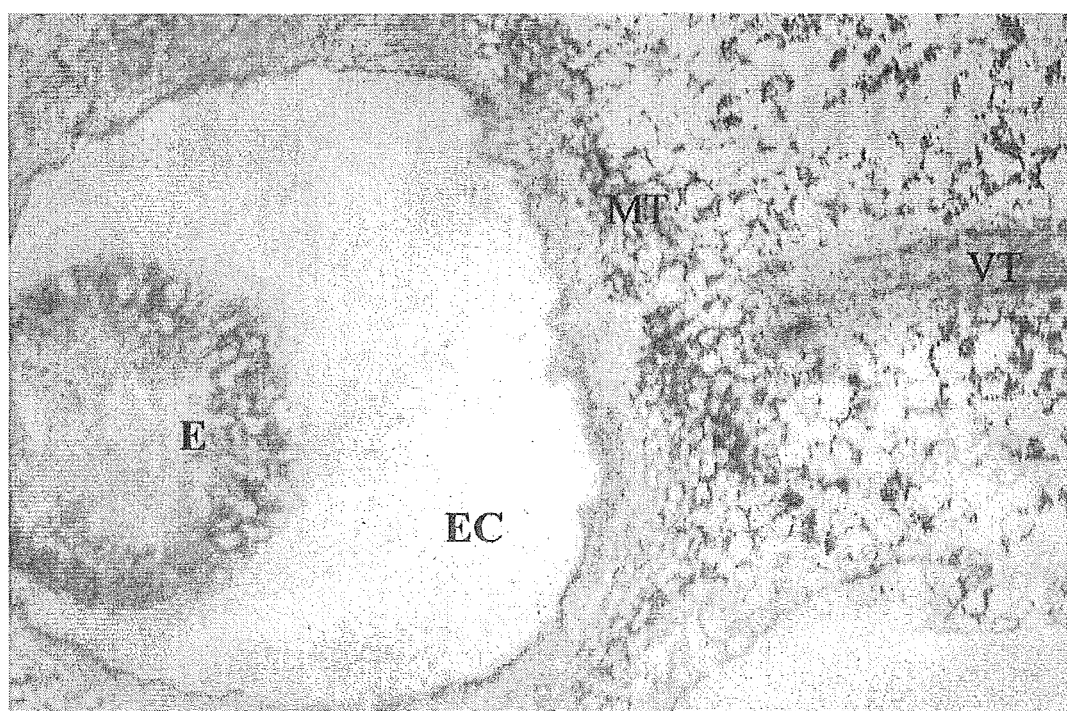
Plates 14-18 show the development of the endosperm 20, 40, 51 and 62 DAP. At 20 DAP the endosperm is only an empty cavity (plate 14) which has yet to be filled with storage products. At 40 DAP the endosperm cavity is starting to fill (plate 15). A small immature endosperm is observed. At 62 DAP the endosperm cavity is completely full, with the endosperm occupying the whole cavity (plate 18).

Plate 17 shows the immature embryo, in the endosperm at 51 DAP. At 62 DAP (plate 18) the embryo is shown to have elongated through cell division.



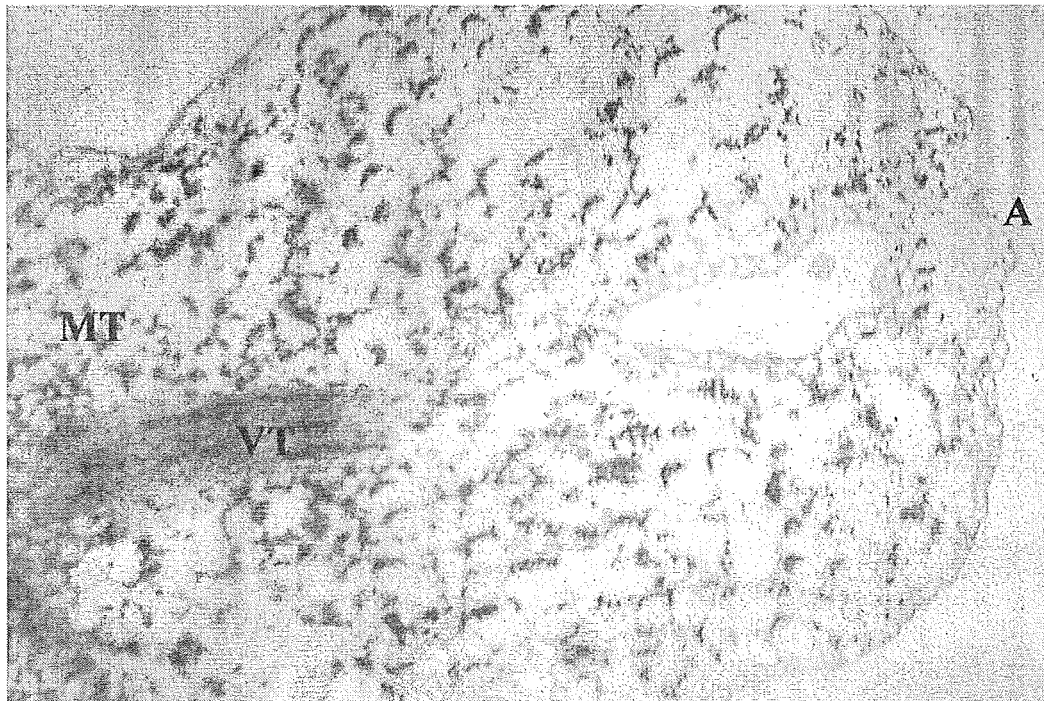
0.1mm

Plate 14. Light micrograph of a seed cross section fixed in resin at 20 DAP, at 40x magnification. **EC**; Endosperm Cavity, **MT**; Mother Tissue, **D**; Differentiated tissue.



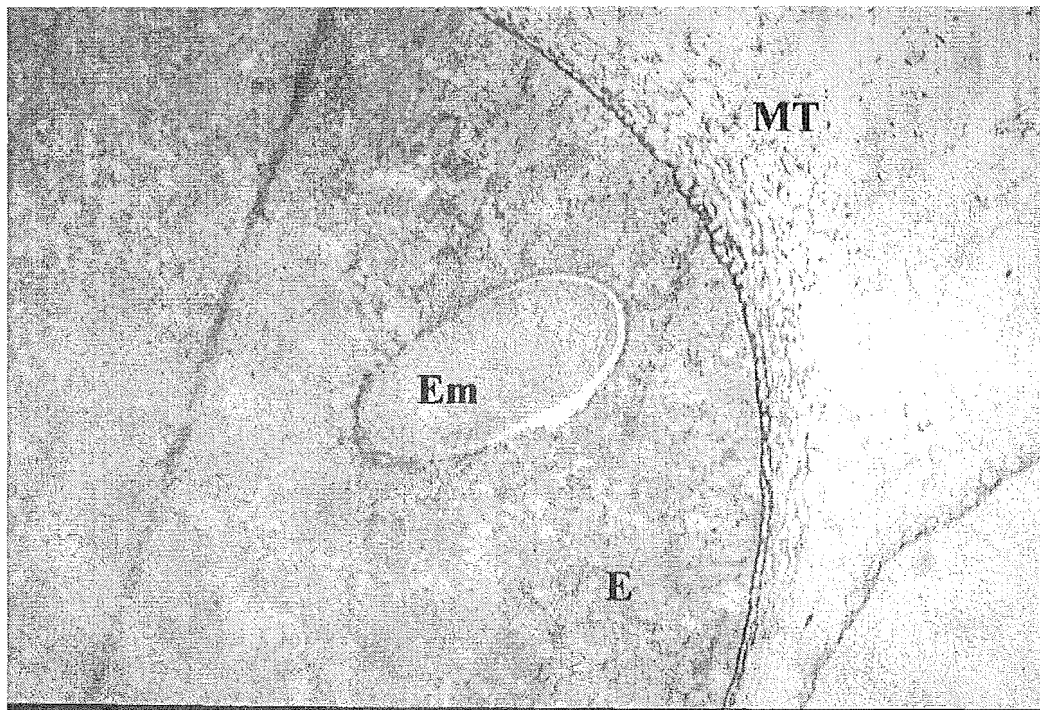
2mm

Plate 15. Light micrograph of a partial seed cross section fixed in resin 40 DAP, at 40x magnification. **E**; Endosperm (immature), **EC**; Endosperm Cavity, **MT**; Mother Tissue, **VT**; Vascular Tissue. Plates 15 and 16 are photographs of different parts of the same seed.



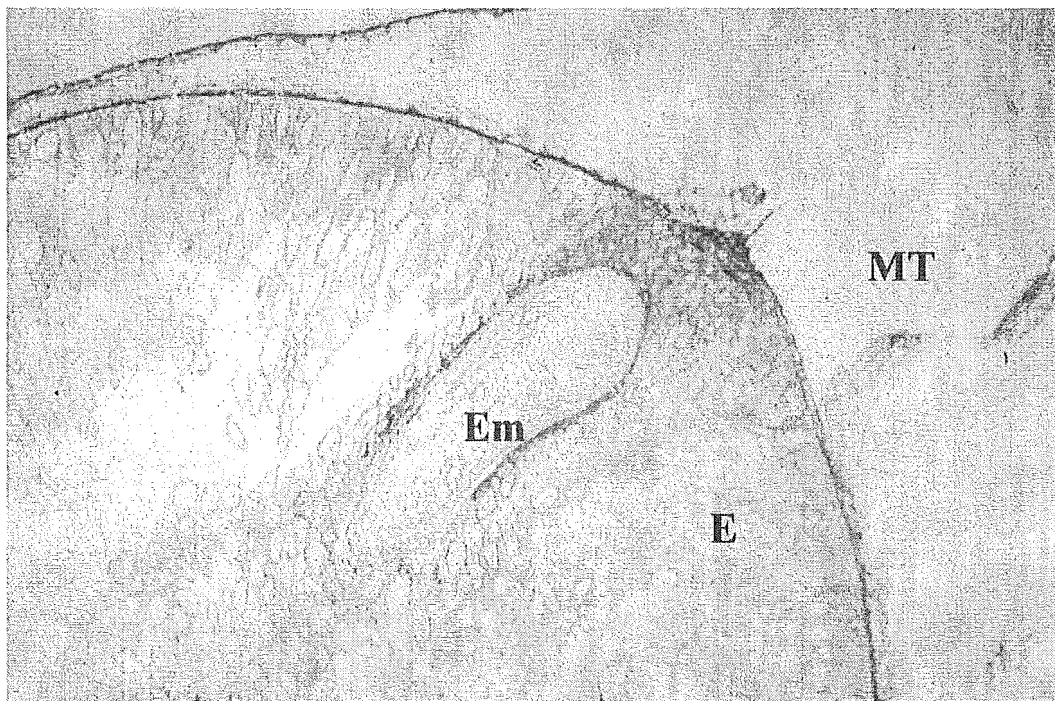
0.5mm

Plate 16. Light micrograph of a partial seed cross section fixed in resin at 40 DAP, at 40x magnification. A; Attachment point of seed to seepod, VT; Vascular Tissue, MT; Mother Tissue.



0.5mm

Plate 17. Light micrograph of a partial seed cross section fixed in resin at 51 DAP, at 40x magnification. **Em**; Embryo, **E**; Endosperm, **MT**; Mother Tissue.



0.5mm

Plate 18. Light micrograph of a partial seed cross section fixed in resin at 62 DAP, at 40x magnification. **Em**; Embryo, **E**; Endosperm, **MT**; Mother Tissue.

3. 6. 2 Transmission Electron Microscopy

3. 6. 2. 1 Protein Body and Cellular Observations

Plates 19-26 show the development of seed endosperm cells 20, 40, 51 and 62 DAP at a cellular level. Also, within the same developmental age, a comparison was made between the inner and outer cells of the endosperm.

Cellular structures identified as protein bodies by Pernollet (1978) and Bewley and Black (1978) are observed in plates 19-26.

At 20 DAP (plates 19 and 20), before the formation of the endosperm ball, both the inner (differentiated) and outer mother tissue cells are densely packed with organelles. There are very few lipid droplets and protein bodies. In plate (19B) at 6000x magnification, only 4 lipid droplets and 7 small protein bodies are immediately visible.

At 40 DAP (plates 21 and 22) there are differences in the cell contents, when compared to the 20 DAP plates. There are also differences between the inner and outer endosperm cells. The outer endosperm cells contain numerous lipid bodies (plates 21 A and B) and partially filled protein bodies. In the surrounding regions (plate 21 A) smaller protein ‘particles’ are visible next to their respective endoplasmic reticuli. The inner endosperm cells (plates 22 A and B) contain a far greater number of lipid droplets and protein bodies. The larger, inner protein bodies appear more dense and full than those in the outer endosperm, and contain globoid inclusions (plate 22 A).

At 51 DAP (plates 23 and 24) the outer endosperm cells are densely packed with lipid droplets (plate 23 B) and some protein bodies containing globoid inclusions (plate 23 A). The inner endosperm cells contain very large, densely packed protein bodies. The protein bodies appear to be more tightly packed than the 40 DAP examples. At 51 DAP plate (24 B) shows the inner endosperm cells

contain larger and more numerous globoid inclusions, than any younger sampled cells.

At 62 DAP (plates 25 and 26) the outer endosperm cell protein bodies appear larger in size, than any previously sampled age, but appear more 'grainy' and less densely packed than the 40 or 51 DAP examples. The same observation is made for the inner endosperm cells (plates 26 A and B). The protein bodies appear very large in size, but slightly less densely packed than the previous age groups'.

A

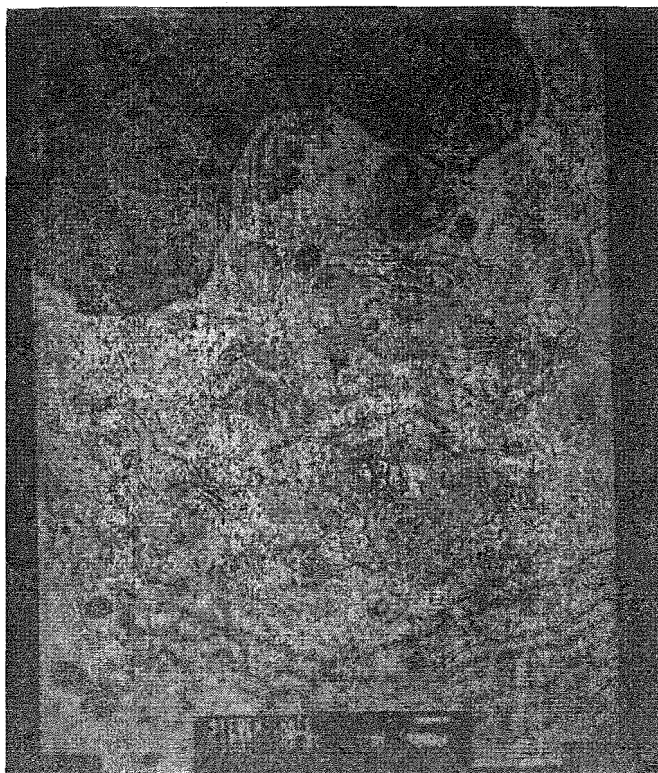


B



Plate 19 A and B. TEM photographs of cells from the outer endosperm tissue of a seed harvested 20 DAP. Magnification 6000X. Scale bar is 1 micrometer. A. V; Vacuole. B. L; Lipid droplet, P; Protein body.

A



B

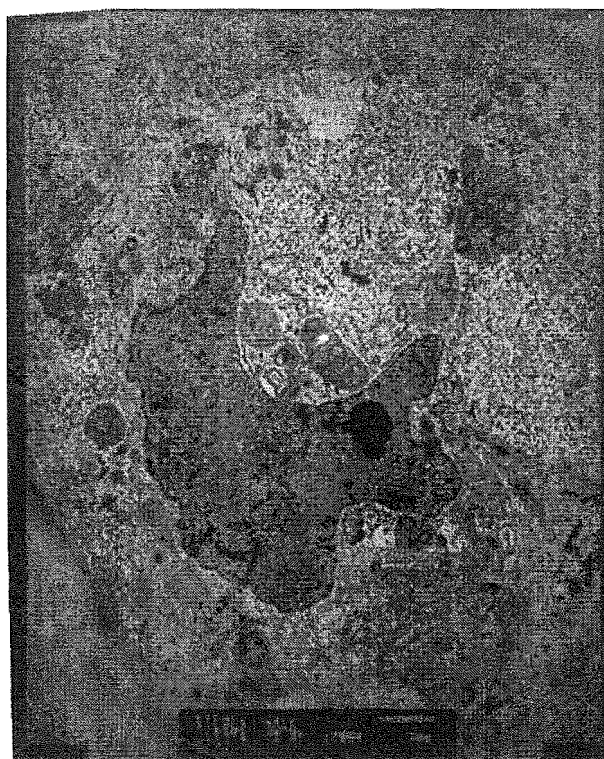
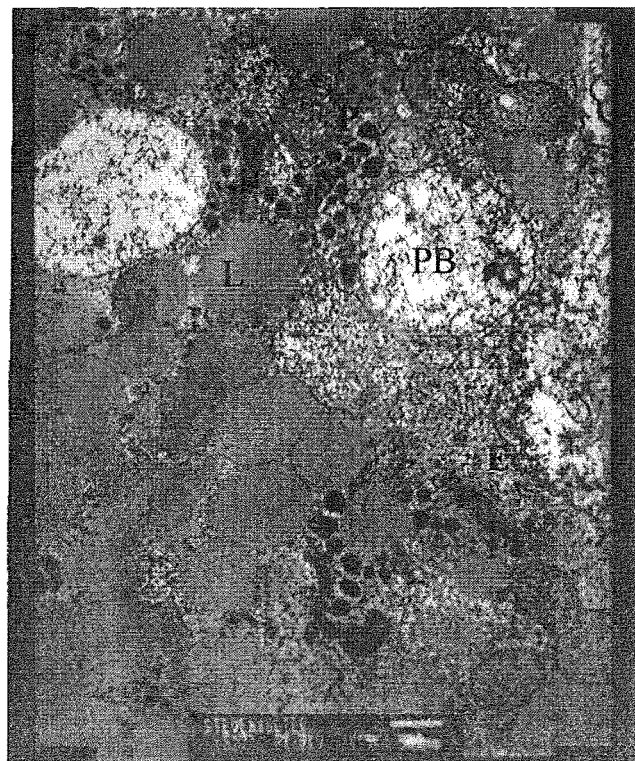


Plate 20 A and B. TEM photographs of cells from the inner endosperm tissue of a seed harvested 20 DAP. Magnification **A**; 10,000X, **B**; 4000X. Scale bars are **A**; 500 nanometers, **B**; 2 micrometers.

A

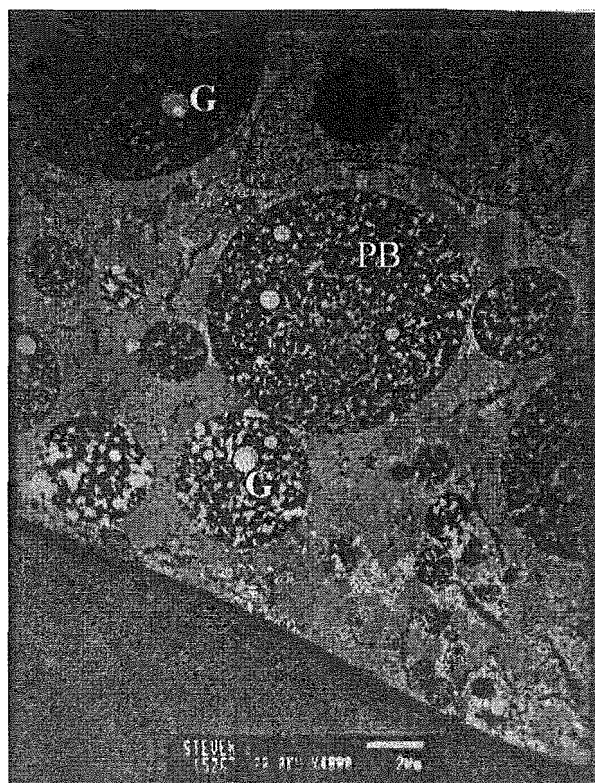


B



Plate 21 A and B. TEM photographs of cells from the outer endosperm tissue of a seed harvested 40 DAP. Magnification A; 15,000 X, B; 10,000 X. scale bar is 500 nanometers. A. L; Lipid droplet, P; Protein particle, PB; Protein Body, E; Endoplasmic reticulum. B. PB; Protein Body, A; Amyloplast.

A



B

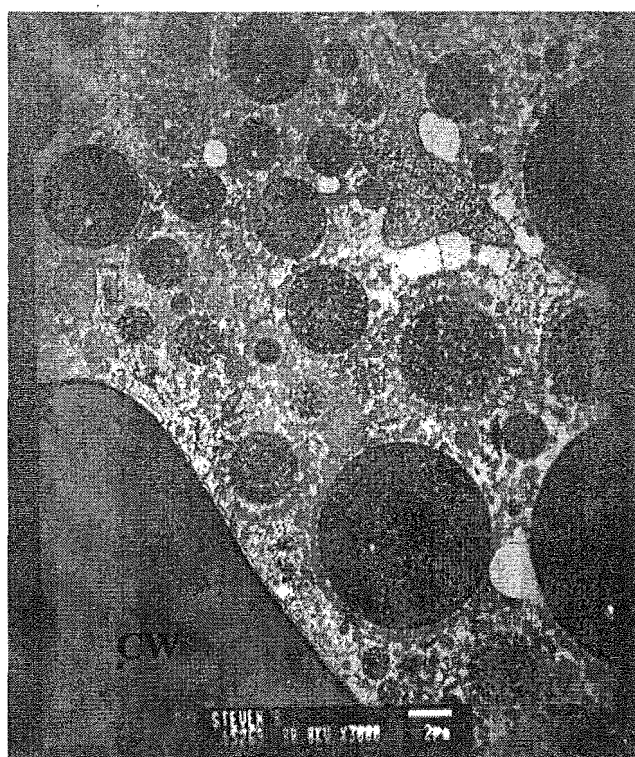
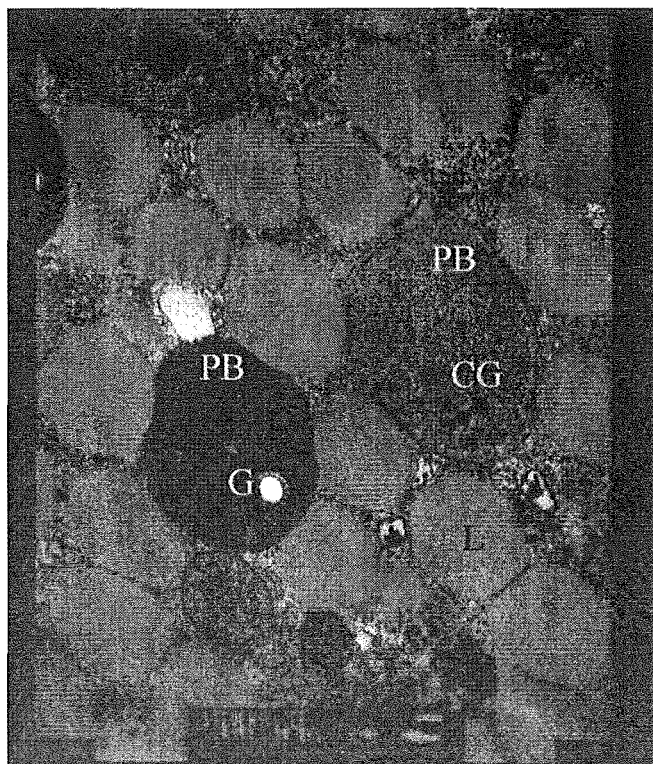


Plate 22 A and B. TEM photographs of cells from the inner endosperm tissue of a seed harvested 40 DAP. Magnification A; 4000 X, B; 3000 X. Scale bar is 2 micrometers. A. PB; Protein Body, L; Lipid droplets, G; Globoid inclusion (soft). B. CW; Cell Wall.

A



B

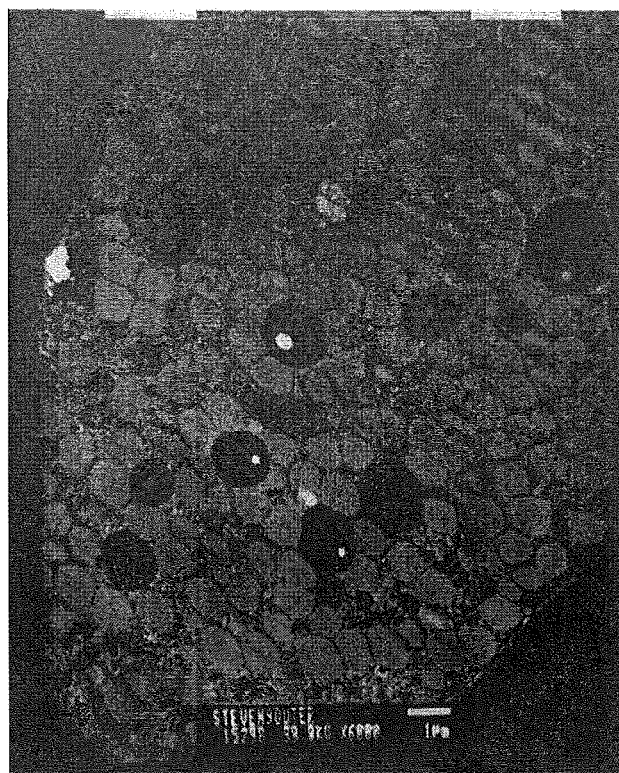
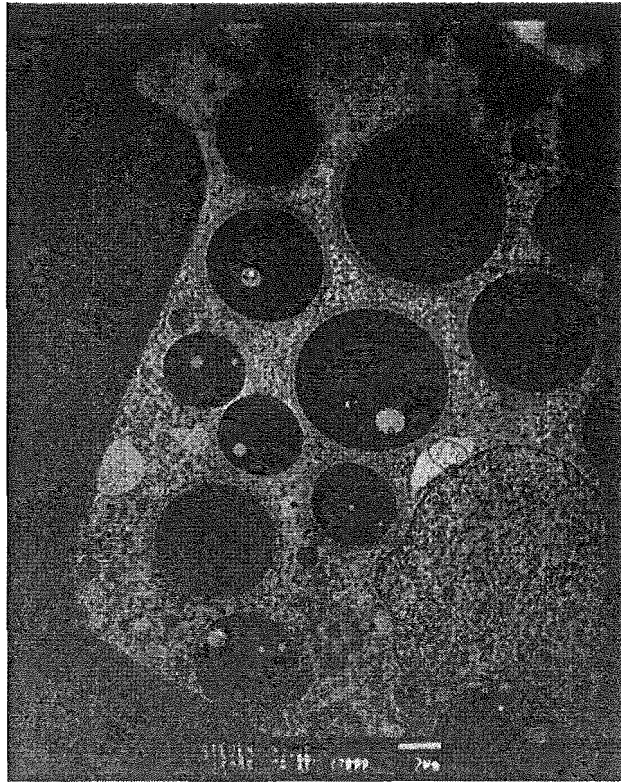


Plate 23 A and B. TEM photographs of cells from the outer endosperm tissue of a seed harvested 51 DAP. Magnification **A**; 20,000 X, **B**; 6000 X. Scale bars are **A**; 200 nanometers, **B**; 1 micrometer. **A**, **L**; Lipid droplet, **PB**; Protein Body, **G**; globoid (soft), **CG**; Crystal Globoid.

A



B

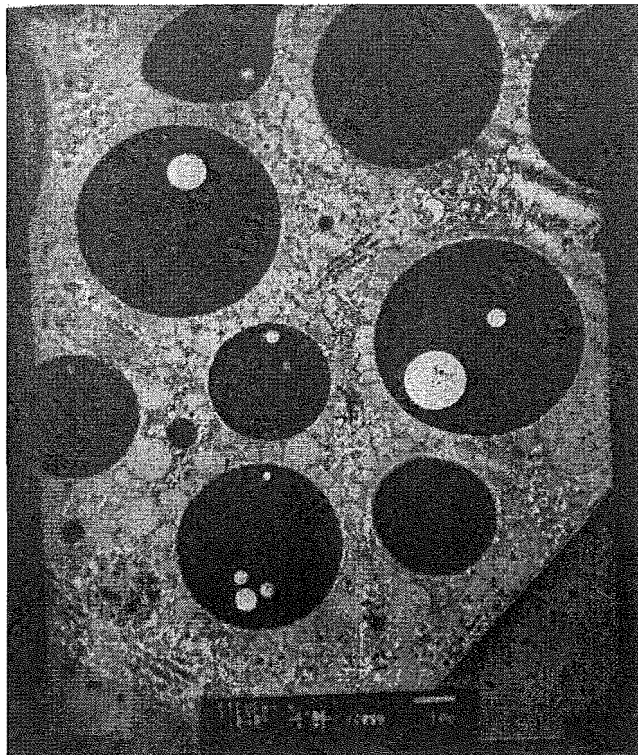


Plate 24 A and B. TEM photographs of cells from the inner endosperm tissue of a seed harvested 51 DAP. Magnification A; 3000 X, B; 6000 X. Scale bars are A; 2 micrometers, B; 1 micrometer.

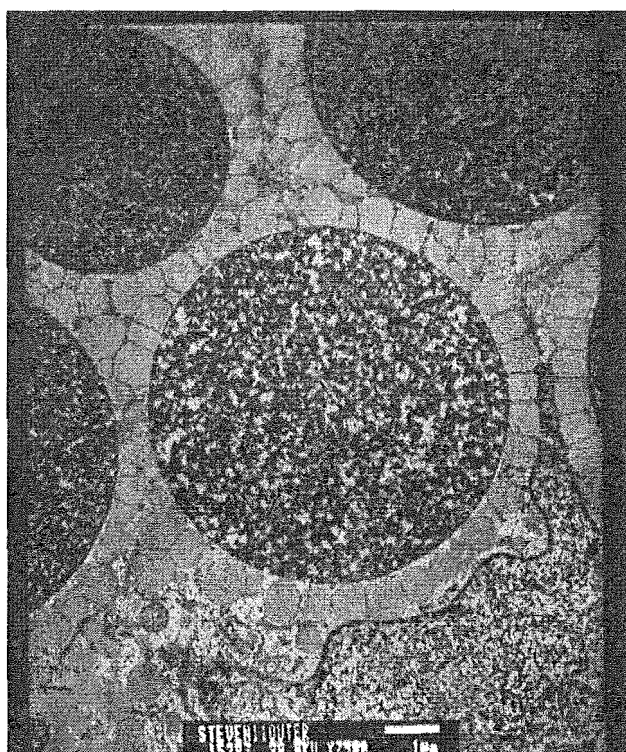
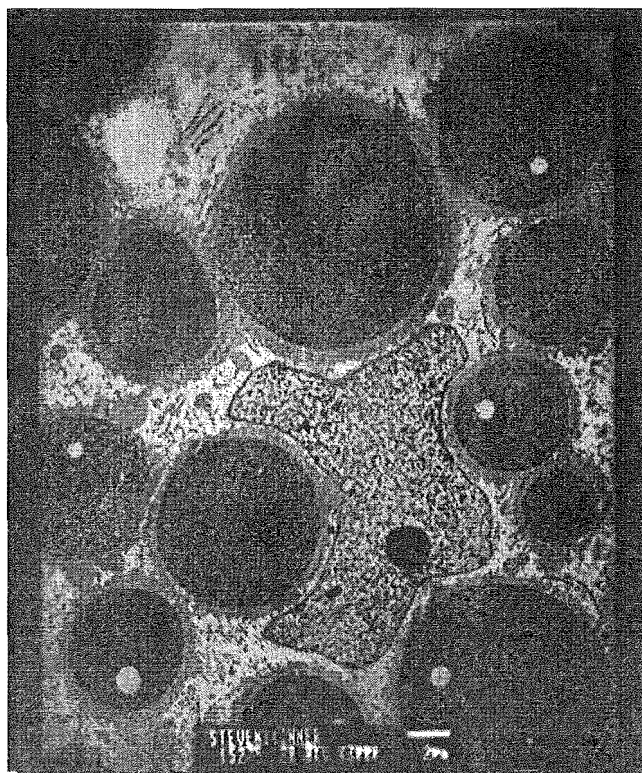


Plate 25. TEM photograph of a cell from the outer endosperm tissue of a seed harvested 60 DAP. Magnification 7500 X. Scale bar is 1 micrometer.

A



B

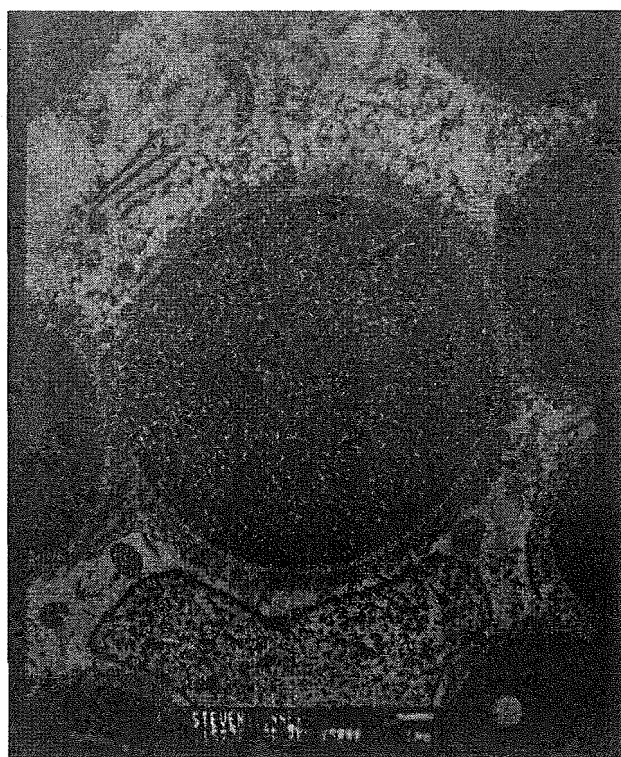


Plate 26 A and B. TEM photographs of cells from the inner endosperm tissue of a seed harvested 60 DAP. Magnification **A**; 3000 X, **B**; 5000 X. Scale bars are **A**; 2 micrometers, **B**; 1 micrometer.

3. 6. 2. 2 Cell Wall Observations

Further observations, at a cellular level, were made regarding cell wall development of seed endosperm tissue. At the sampled seed ages (20, 40, 51 and 62 DAP) differences were noted in the appearance of the endosperm tissue cell walls. Cell wall development as described in Raven *et al* (1992) is shown in plates 27-32.

Plates 27 A and 29 A show that the non-endosperm outer cells of the seed coat contain starch amyloplasts (plate 27 A) and starch granules (plate 29 A). Starch granules were observed in the non-endosperm outer cells at each of the sampled ages.

At 20 DAP, plates 27 B and 28 show early formation of the primary cell wall. Deposition of cell wall precursors (starch and waxes) from the cell (plate 27 B) to the 'almost complete' cell wall is visible. Plate 28 shows several cells beginning to form wax (cutin, suberin) layers around their respective cells.

At 40 DAP, plates 29 B and 30 show the completion of the primary cell wall surrounding seed endosperm cells. The 'stripped' appearance of the cell wall is due to the deposition of alternating layers of wax, to prevent cell water loss during seed desiccation. In both plates small intercellular spaces are visible.

At 51 DAP plate 31 shows the intercellular spaces within the cell wall have disappeared altogether due to the dense deposition of cell wall waxes, for the seed desiccation phase.

At 62 DAP plate 32 shows the cell wall formation is complete, creating a large structural barrier between cells. Plate 32 B shows a narrowing of the cell wall between neighbouring cells which may be a primary pit field. Close inspection of the plate reveals a transfer of particles between the cells.

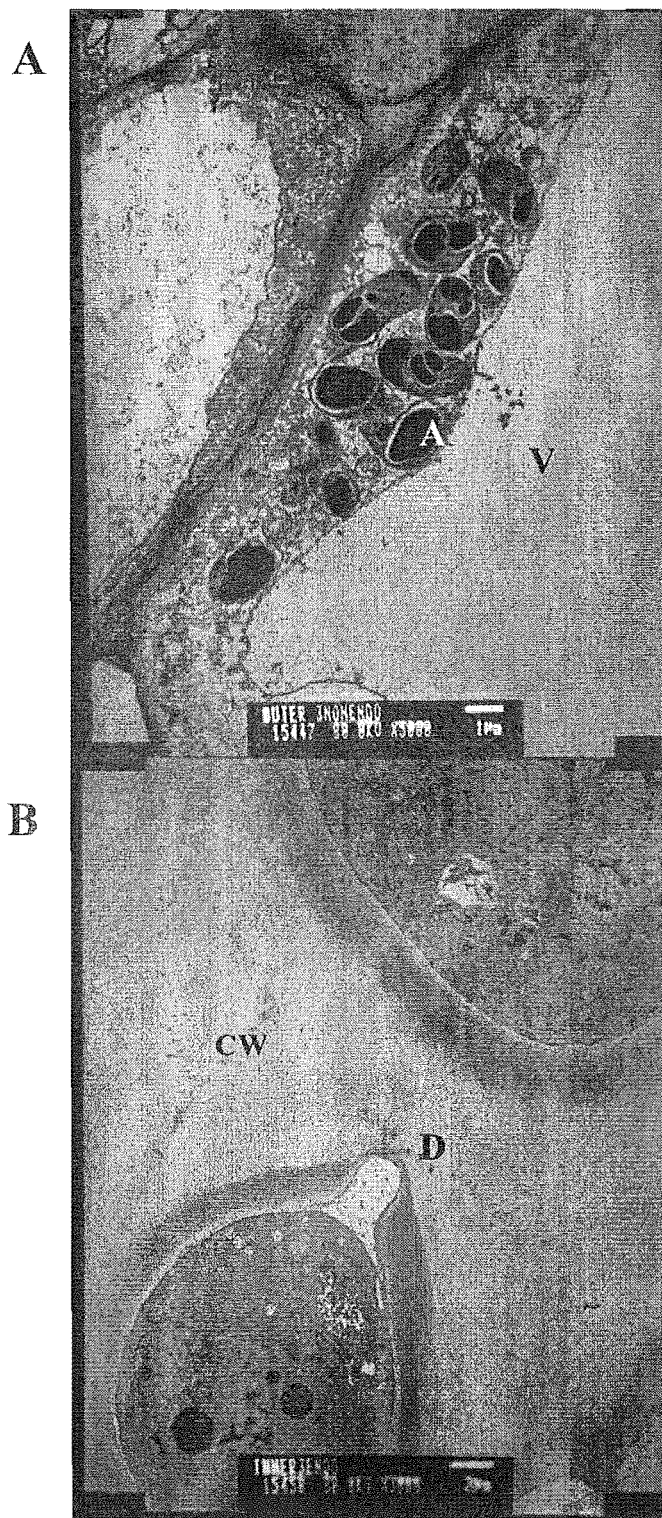
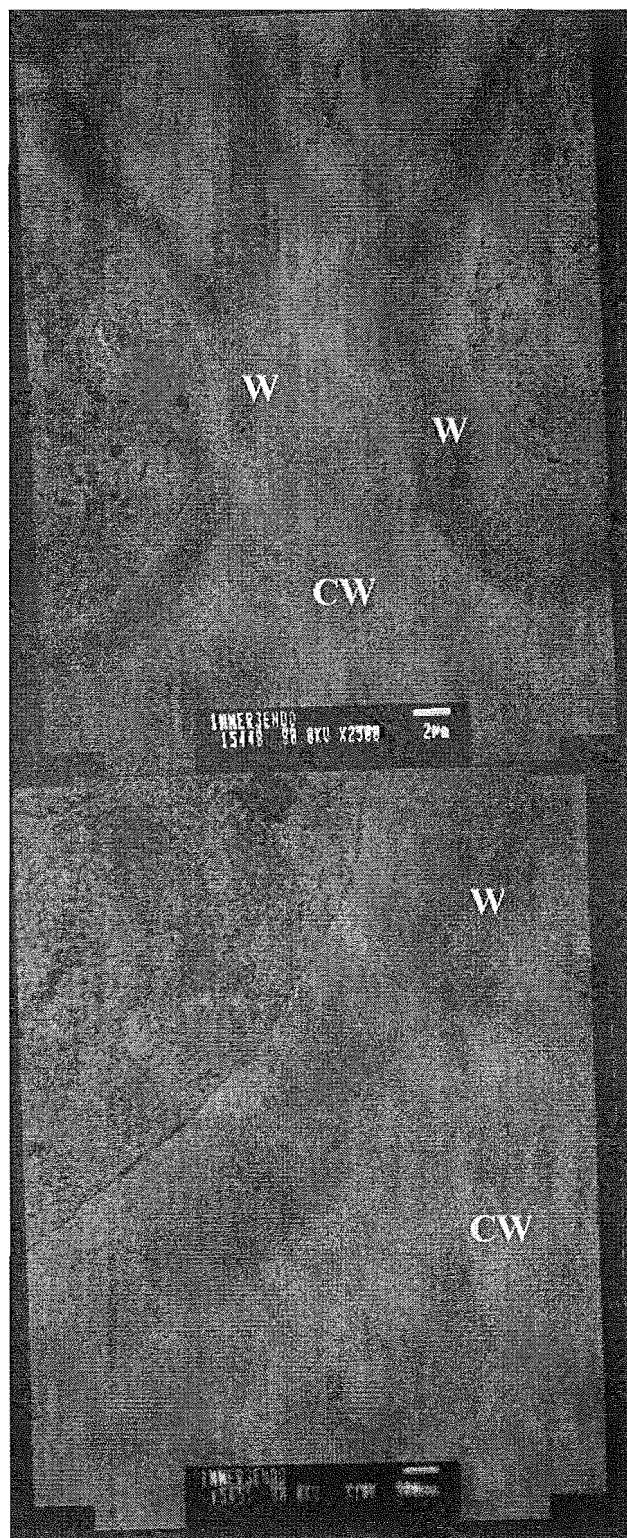


Plate 27 A and B. TEM photographs of cells from the non-endosperm (A) and inner endosperm (B) tissue of a seed harvested 20 DAP. Magnification A; 5000 X, B; 3000 X. Scale bars are A; 1 micrometer, B; 2 micrometer. A. A; Amyloplast, V; Vacuole. B. CW; Cell Wall, D; Deposition of cell wall components.

A



B

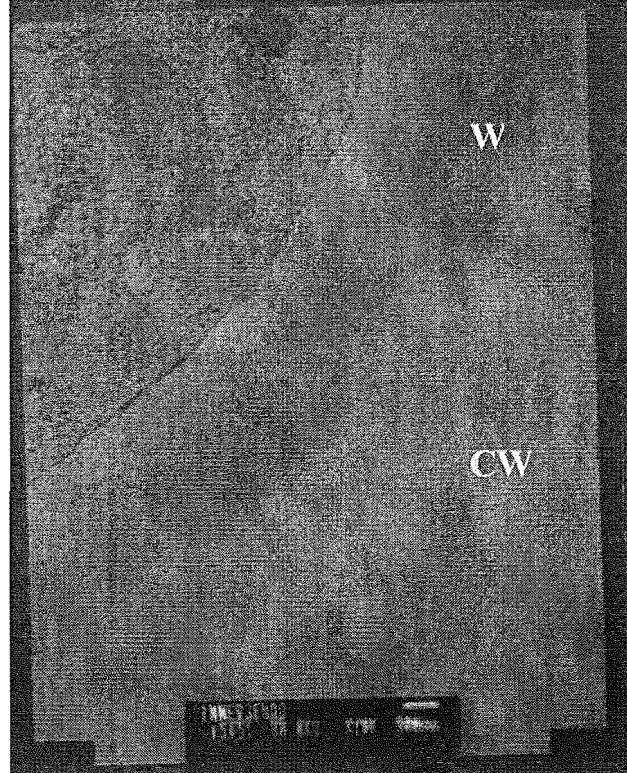


Plate 28 A and B. TEM photographs of cells from the inner endosperm tissue of a seed harvested 20 DAP. Magnification **A**; 2500 X, **B**; 10,000 X. Scale bars are **A**; 2 micrometers, **B**; 500 nanometers. **A** and **B**, **CW**; Cell Wall, **W**; Wax.

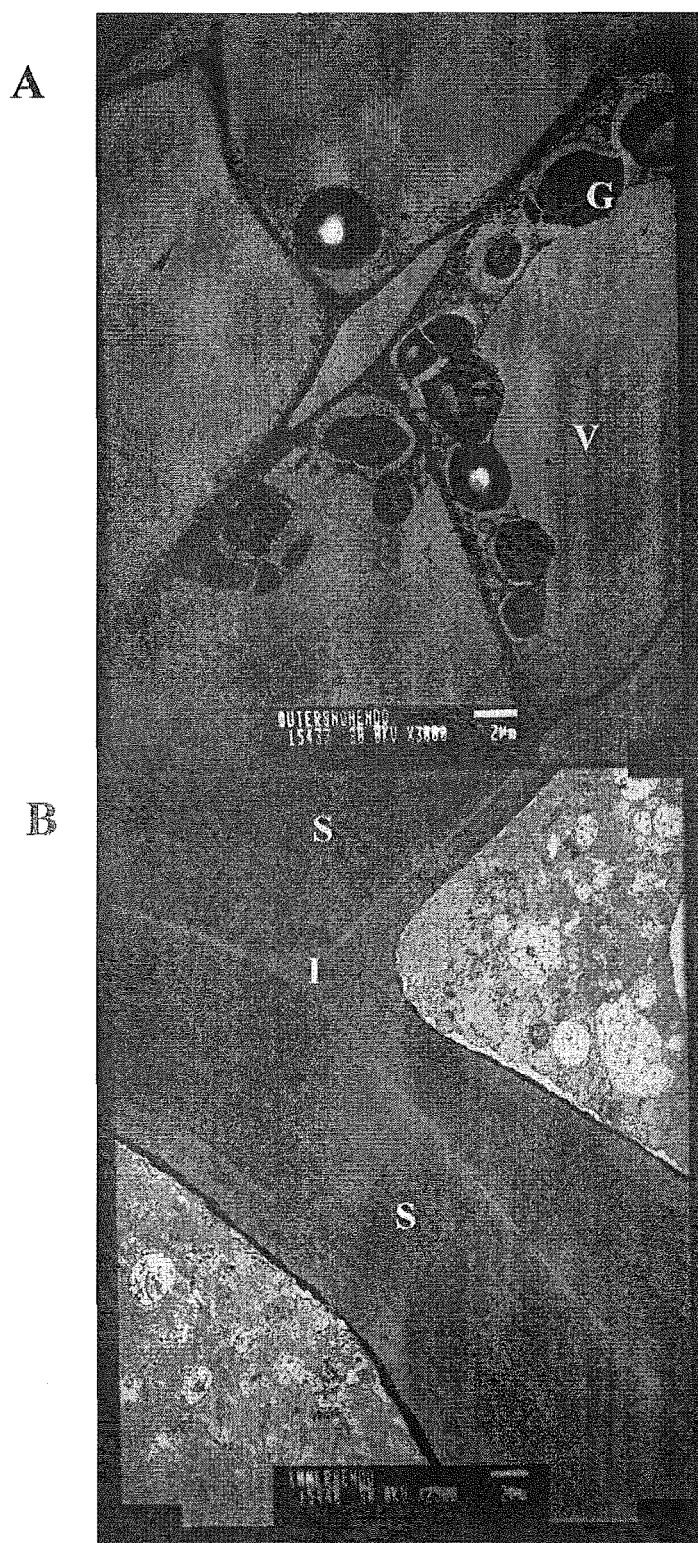


Plate 29 A and B. TEM photographs of cells from the non-endosperm (A) and inner endosperm (B) tissue of a seed harvested 40 DAP. Magnification A; 3000 X, B; 2500 X. Scale bars are 2 micrometers. A. G; Granule of starch, V; Vacuole. B. I; Intercellular space, S; primary cell wall 'Stripping'.

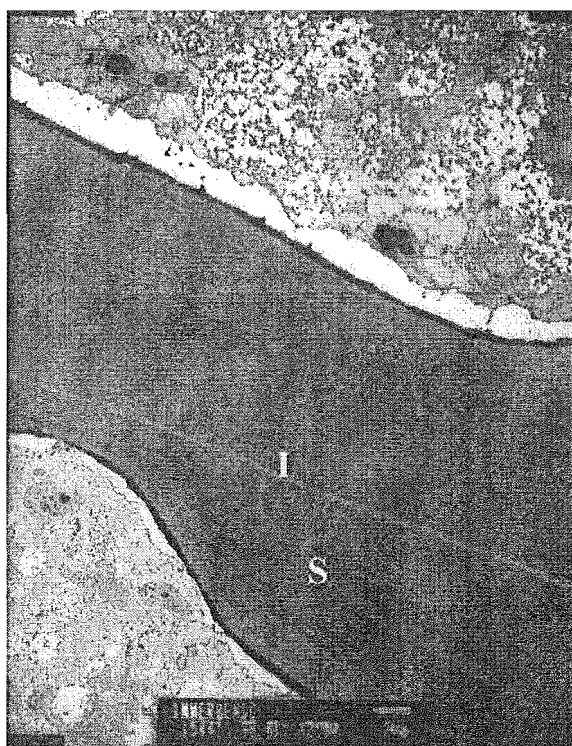


Plate 30. TEM photograph of the cell wall of cells from the endosperm tissue of a seed harvested 40 DAP. Magnification; 2500 X. Scale Bar is 2 micrometers. **I**; Intercellular space, **S**; primary cell wall 'Stripping'.

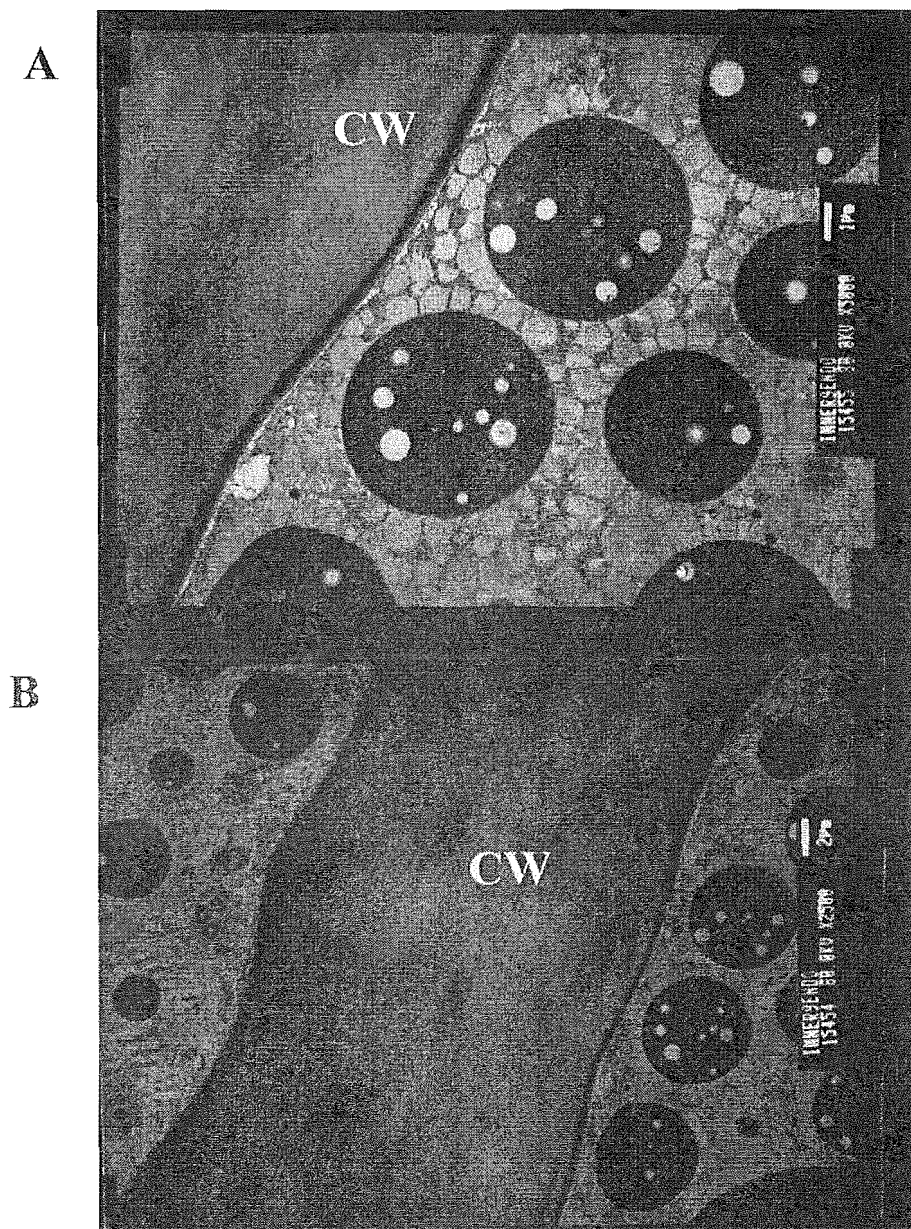
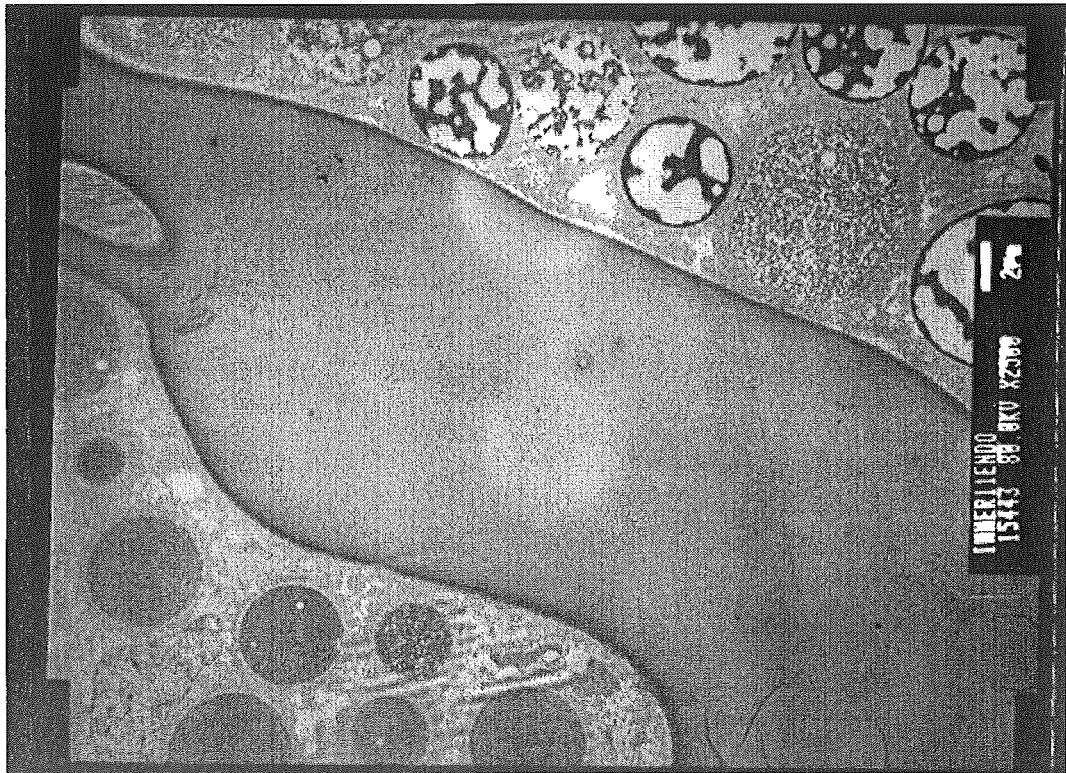


Plate 31 A and B. TEM photographs of cells from the inner endosperm tissue of a seed harvested 51 DAP. Magnification **A**; 5000 X, **B**; 2500 X. Scale bar is **A**; 1 micrometer, **B**; 2 micrometers. **A and B.** CW; Cell Wall (containing wax 'striping').

A



B

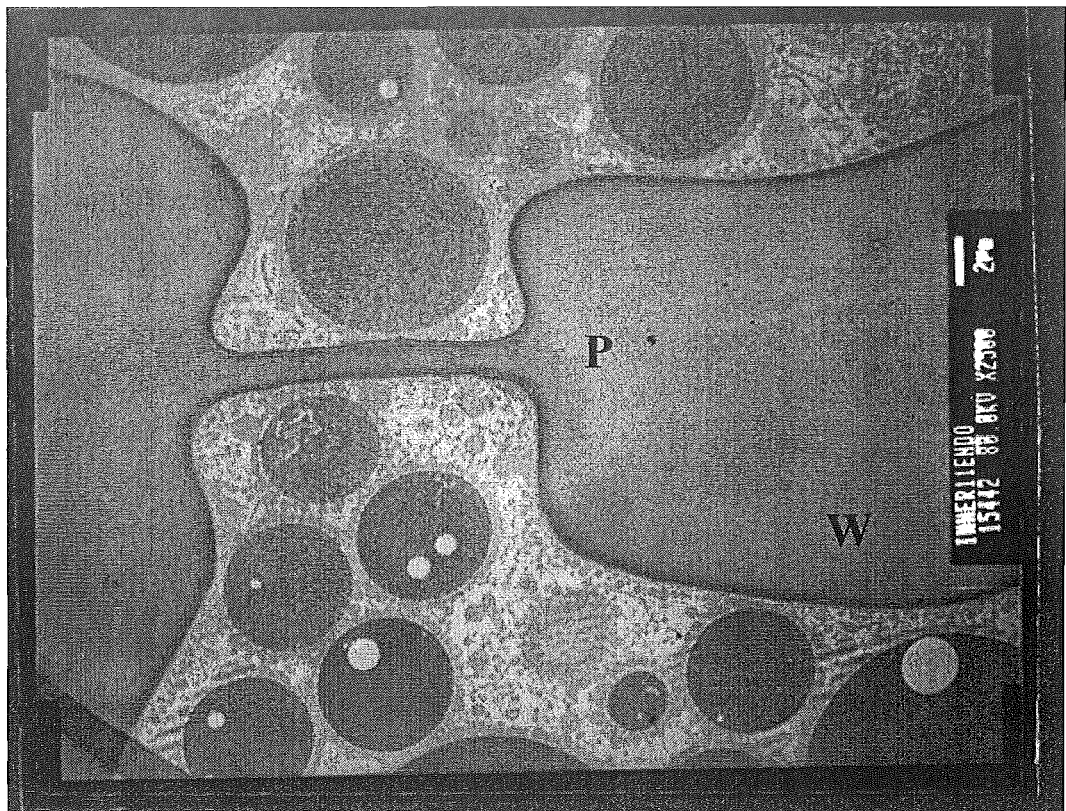


Plate 32 A and B. TEM photographs of cells from the inner endosperm tissue of a seed harvested 62 DAP. Magnification A and B; 2500 X. Scale bars are 2 micrometers. B. P; Primary pit field, W; Wax 'striping'.

3. 7 Germination Tests

Two series of germination tests were carried out. The less formal, first test involved the use of filter paper as a medium and a 16 hour photoperiod growth room. The seeds were kept in darkness, covered by a plastic ice cream container. After 4 weeks all petri dishes became contaminated with fungi. One seed germinated and was transferred to PPM antifungal solution. A small shoot formed but the entire seed became infected. Plate 1 shows the germinated seed.

A second germination test involved 200 mature seeds soaked overnight in distilled water, sealed in autoclaved glass petri dishes with germination paper. The dishes were put in a 24°C constant darkness incubator room. After 4 weeks none of the seeds germinated.

3. 8 Embryo Detection

Initially 30 seeds (from the same crop) were dissected but no embryos were observed. At the time of dissection the seeds ultrastructure was less well understood. After the fixation and light microscopy sections of research several more seed dissections took place.

Fifty randomly selected seeds from crop 2 were soaked in distilled water over night. Approximately half the seeds floated and the other half sank. Of 25 sunken seeds dissected, only 1 did not contain an embryo. It is interesting that a small number of embryos appeared transparent and not opaque like the majority. Twenty floating seeds were also dissected. All were found to contain embryos. Seeds randomly selected from other crops were also dissected, with the majority containing embryos. It was noted though, that approximately half the embryos observed appeared transparent, resembling endosperm tissue, and not opaque like distinct structures. Also a high number of these seeds had an unfilled cavity adjacent to the embryo.

CHAPTER 4

DISCUSSION

4.1 Extraction of Seed Proteins

Dating back to the 1800s (Spencer, 1995), the study of seed proteins is not a new field, though it is in relation to the seed storage proteins from non-agricultural, ornamental plants, such as *Sandersonia*. Due to this lack of previous research, the protein extraction methods used, in a thorough study of castor bean storage proteins (Gifford *et al*, 1982), were trialled for the present study.

Plate 2 compared the salt soluble proteins (presumably enzyme and protein body matrix proteins) extracted using phosphate buffer and any detergent soluble proteins extracted using the SDS PAGE sample buffer, after separation under denaturing electrophoretic conditions. Gel lanes were standardised by loading protein extracted from multiple samples of equal fresh weight. Although both buffers extracted the same six protein bands (approximately 54.9, 51.9, 36.3, 32.5, 20.9, and 17.2 KDa), the sample buffer bands appeared more intense. This increased intensity may be due to the SDS detergent in the sample buffer. The strong detergent action may extract a greater quantity of the same proteins. When sequentially re-extracting a single pellet with the same buffer type, the entire quantity of protein is not completely solubilised. Decreasing amounts of protein may be extracted from the same pellet by a limited number of subsequent re-extractions. The difference in gel intensity between buffers may be due to the sample buffer extracting a greater quantity of the same protein and not necessarily any other proteins that are not salt soluble. Despite the differences in staining intensity, the gel demonstrates that mature *Sandersonia* seeds contain salt-soluble, globulin type proteins, as defined by Osborne (see Higgins, 1984).

Plate (3) shows a comparison of the salt soluble proteins of mature seeds and the proteins extracted by a CPE (Crystalloid Protein Extraction) buffer with, and without mercaptoethanol. The gel image shows that CPE buffer extracts the same 6 protein bands. This demonstrates that mature *Sandersonia* seeds contain only globulin (salt-soluble) proteins, the subunits of which aren't bound by disulphide bonds or contain intra-chain disulphide bonds. By default, it must be concluded that the subunits are bound by non-covalent forces and not disulphide bonds. The gel also shows that no crystalloid proteins are present in the protein body matrices.

Comparison of the salt soluble (globulin) and water soluble (albumin) seed proteins under denaturing electrophoretic conditions suggested that the major *Sandersonia* seed proteins are globulins. To positively demonstrate that the major seed proteins do not contain an albumin fraction, the phosphate buffer extracted protein could be dialysed extensively against water to determine whether the protein remains soluble, as per the method of Morcillo *et al* (1997).

Together, the results in plates 2,3 and 4 suggest that the globulin proteins present in mature *Sandersonia* seeds resemble the vicilins in the following aspects:

1. The protein subunit (band) size range corresponds to the known vicilin range, 20-56 KDa (Bewley and Black, 1978);
2. The cumulative subunit molecular mass (approximately 213.7 KDa) is close³ to the vicilin range, 180-200 KDa (Spencer, 1984);
3. The inter-subunit bond forces match those generalised for vicilins: non-covalent, not separated into further subunits by mercaptoethanol (Shewry, 1995 and Spencer, 1984); and
4. The six SDS PAGE bands may make up three pairs of subunits, possibly forming the common trimeric vicilin structure (Shewry, 1995).

³ The estimated cumulative subunit molecular mass may be heavier than the actual native protein due to the standard curve used in the calculation. The calculated 'line of best fit' falls above the plotted values for some mid range data points. Calculated molecular masses of unknown proteins using this 'line of best fit' (equation) are falsely increased.

Marcone *et al* (1998) carried out a study on the globulins of a broad range of food related monocotyledonous and dicotyledonous plants. It was found that the 11S legumins (300-370 KDa), of some plants, did not contain disulphide bridge bound subunits (covalent forces). This demonstrates that the lack of disulphide bonds is not necessarily restricted to vicilin-like globulins. This conclusion has little effect on the interpretation of the present study, as three other (above mentioned) vicilin-like properties are observed in *Sandersonia* seed globulins.

Luthe (1992) and Marcone *et al* (1998) both relied only on subunit molecular masses, extracted in conjunction with mercaptoethanol, to define the types of globulins present in their study plants. In each study, for the legumin-like globulins (~60 KDa subunits) to be separated into basic and acidic subunits (20-27 KDa and 30-39 KDa, respectively), the addition of mercaptoethanol was required. This criterion was used to identify legumin-like globulins, over vicilin-like globulins. In the present study, extraction of the salt soluble proteins with phosphate buffer (containing no SDS or mercaptoethanol) was sufficient to extract all 6 subunits (bands). Extraction with buffers containing SDS and mercaptoethanol revealed no new subunits. Therefore under their definition, the globulin in *Sandersonia* seeds are of the vicilin-like type

Further research, to positively identify *Sandersonia*'s seed proteins as vicilins, could involve native, non-denaturing electrophoretic analysis. In the present study the 6 subunit band masses were added together to estimate the whole protein size. This relied on an assumption that each of the native protein subunits is only present singularly in the whole protein structure. This approach cannot be used when studying legumins, as the six 60 KDa subunits would only be visible as one SDS PAGE band. Therefore the unconfirmed cumulative subunit molecular mass can only be tentatively discussed. Native electrophoresis is needed to establish the whole, non-denatured proteins molecular mass, thereby distinguishing it as fitting into one of the two characteristic size ranges (180-200 KDa; vicilins, or 300-370 KDa; legumins). Another method of establishing the type of globulin present is analytical centrifugation (measured by sedimentation coefficient (S)), separating the globulins as either 7-8S (vicilin) or 11-12S (legumin). It would also be

interesting to carry out two dimensional gel electrophoresis, the first dimension being SDS PAGE and the second isoelectric focusing (IEF), to determine if charged subunits are present (a legumin characteristic).

4. 2 Extraction of Tuber Proteins

The same set of protein extraction buffers used in the seed protein study were used to extract *Sandersonia* tuber proteins. It was found that the major tuber proteins are water soluble albumins (plate 5). Under denaturing electrophoretic conditions (SDS PAGE), the major proteins consist of 4 bands covering a restricted low molecular mass range (plate 6 and table 1).

Also visible on plate 5, are several minor tuber protein bands. These have mid range molecular masses and appear to be differentially extracted between buffers. Nevertheless, none of the major or minor tuber proteins coincide in molecular mass or solubility type with the major seed proteins.

Sandersonia major tuber proteins share little in common with the other previously studied tuber storage proteins. Unlike *Sandersonia*'s major tuber proteins, patatin, sporamin and dioscorin are globular proteins, consisting of linked subunits (Shewry, 1995). Under denaturing electrophoretic conditions (plates 5 and 6), *Sandersonia*'s major tuber proteins manifest as 4 gel bands. A useful extension to the present study, would be to investigate the nature of the tuber proteins by native, non-dissociating electrophoresis. This technique would confirm whether the major tuber protein bands are subunits of a larger native protein.

As seeds and tubers are both reproductive propagules, each storing nitrogen, carbon and sulphur reserves for new shoot growth (Shewry, 1995), a hypothesis formulated at an early stage of the present study, was that seeds and tubers share the same storage proteins. The molecular mass differences between seed and tuber proteins (plate 6) show that in *Sandersonia* this is not the case. This finding was not totally unexpected though. Apart from the electrophoretic evidence of the present study, a review of literature on the topic provides more evidence against the hypothesis.

Bevan *et al* (1993) stated that, in general seed and tuber proteins are from individual protein classes: the zygotic and somatic protein classes, respectively. Different genes control the two protein classes. Despite this difference, gene expression of the two protein classes is both controlled by metabolite levels during expression. Shewry (1995) contrasted the relative homology of biochemical structure of seed proteins with the diversity of biochemical structure of tuber proteins. It was also stated that seeds in general are similar anatomical structures, whereas tubers are a congregation of diverse anatomical structures (swollen stems and roots).

Although *Sandersonia* seed and tuber proteins superficially serve a similar function within the plant, their solubility type and subunit sizes are not, and apparently, do not need to be the same to carry out this function. Apart from supporting the vegetative reproduction of plants, the major tuber proteins examined in other studies, exhibit secondary activity, for example, trypsin inhibition by sporamin of sweet potato (Conlan *et al*, 1997; Shewry, 1995). With this in mind, *Sandersonia*'s major tuber proteins may have some other function, which is responsible for the difference in solubility type, when compared to the major seed proteins.

4. 3 Seed Storage Protein Development Study

4. 3. 1 SDS PAGE Gels and Seed Germination Test

As stated in section 1. 7, seed storage proteins can be defined as “any protein accumulated in significant quantities in the developing seed which on germination is rapidly hydrolysed to provide a source of reduced nitrogen for the early stages of seedling growth” (Higgins, 1984). For a major seed protein to be called a storage protein this definition must apply, with respect to its accumulation and breakdown. With this in mind, the development of *Sandersonia* major seed proteins was monitored from 10 to 60 days after pollination (DAP), to confirm the presence of true storage proteins. Plates 7 and 8 show, that the major seed proteins form by 30

DAP, but not before 25 DAP. The characteristic 6 band electrophoretic pattern appears at 30 DAP. Formation of the major seed protein bands at approximately 30 DAP is also observed in cowpea and castor bean seeds (Khan *et al*, 1980 and Gifford *et al*, 1982, respectively).

Between 30 and 60 DAP the *Sandersonia* seed bands increase in intensity, demonstrating an accumulation of protein. This fulfils the first half of the storage protein definition.

In order to observe the breakdown and utilisation of the major proteins, and to meet the second half of the definition, seed germination tests were carried out. The intention was to observe a time course of the degradation of the major seed proteins using SDS PAGE analysis. Due to a deep dormancy mechanism, it was not possible to germinate enough mature seeds for this analysis. Only one seed germinated, giving indirect evidence of the successful degradation of the major seed proteins. But, without a gel demonstration of protein degradation, it can only be tentatively concluded that the major proteins of *Sandersonia* seeds are storage proteins. This conclusion relies on the assumption that the accumulated proteins breakdown during germination. Though this assumption is not unrealistic, it still requires experimental verification. Given a longer research period, through leaching and chilling (Warren, 1988 and Clark, 1994, See Appendix 2) the deep dormancy mechanism may be broken, and the degradation of major seed proteins demonstrated. This would meet the second half of the storage protein definition.

4. 4 Tuber Storage Protein Development Study

As previously described, the majority of research on tuber storage proteins has involved in depth investigation of the major proteins of economically important food related tubers, such as yams and potato (Conlan *et al*, 1997; Desborough, 1985; Harris, 1978; Paiva *et al*, 1983; Park *et al*, 1983; Shewry, 1995). To date, there have been no published studies relating to the time course of major tuber protein degradation as a comparison to the present study.

4. 4. 1 Initial Tuber Planting

Seven 15 to 20g size mother tubers (as defined by Ijiro and Ogata, 2000) were planted and one harvested each week for six weeks. The first tuber was sampled with out planting. The tubers were planted and grown under identical conditions, to examine a time course of storage protein degradation, during the first six weeks of new season growth. At the time of weekly harvest, the mother tuber was excavated and its tissue sampled in three anatomical locations (see plate 9).

Brooking *et al* (1997) found that daughter tuber growth rates are strongly affected by temperature and irradiance. Due to the short length of the study (7 weeks during early summer), the effects on daughter tuber growth, due to changes in seasonal temperature, were assumed to be negligible in the present study.

4. 4. 2 SDS PAGE Gels

Within the first six weeks of new season tuber growth, at each of the sampled tissue locations, no visually significant difference was noted in SDS PAGE banding of the major mother tuber proteins (plates 10, 11 and 12). Neither the intensity nor the molecular mass migration of the major tuber protein bands changed during the sampling period.

Brundell and Reyngoud (1985) found that during the first six weeks after planting there were two major growth 'events'. After approximately four weeks a shoot emerges and grows quickly. Also during this period, daughter tuber growth is initiated, and is visible as a tuber when the tenth leaf unrolls, approximately 3 weeks after shoot emergence (Ijiro and Ogata, 2000).

At an early stage in the present study, a hypothesis was held that the mother tubers major proteins would be used and depleted during the period of shoot emergence. For shoot emergence to occur, the mother tuber storage protein must be used, as at that point there are no leaves present to sustain growth.

The gel images show that there are no qualitative or quantitative changes in gel banding during this time. Therefore the amount of protein used by *Sandersonia* mother tubers to sustain shoot emergence and growth, must be minimal compared to the amount required for other possible tuber functions. To examine the possible depletion of major tuber proteins during flowering, seed formation and daughter tuber formation, a further study was carried out. Mother tubers were planted and harvested at 3 further developmental (see later part of section 3. 5. 1) stages. The tubers were again sampled in 3 anatomical locations (see plate 9).

The gel image (plate 13) shows that the formation of immature flowers and the subsequent maturation of half the flowers present has little effect on the electrophoretic appearance of the major tuber proteins, regarding band intensity or band migration.

It is only at the final developmental stage sampled (post-flowering and bearing immature seed pods) that any differences are noted in the major proteins electrophoretic appearance. There are no differences noted in the four tuber storage protein bands from the daughter tuber (developmental stage), through to the pre-flowering stage. It is only during at the mid and post flowering stages that any difference is noted in the mother tuber storage proteins. At each of the three sampled locations the major tuber proteins are heavily depleted and not visible on the gel. Between the last two sampled growth stages there are large developmental differences (increases in plant height, numbers of flowers, seed formation and final formation of the daughter tuber), which were correlated with the depletion of the major proteins. Initial daughter tuber formation (which begins immediately after shoot emergence, Brundell and Reyngoud, 1985), rapid stem growth and flower formation have little effect on the gel banding characteristics (plates 10, 11 and 12). Therefore, it seems likely that the depletive utilisation of the major tuber proteins, corresponds with seed formation, final formation of the daughter tuber, or any secondary tubers.

At an early stage of this study, a hypothesis was held that the storage proteins of seeds and tubers were the same (regarding solubility type and molecular mass) or in some way related. Though this theory was largely disproven in section 4. 3, the

implication that the major tuber proteins are consumed during seed formation, may explain why their similarity is not necessarily required.

The fact that the major proteins of seeds and tubers are different in solubility type and molecular mass may help to identify their origin and use. The water soluble albumin tuber proteins may be solubilised and transported to the developing seedpods. Once near the location of the seeds, the tuber proteins may be modified and permanently deposited as salt soluble globulin storage proteins. This theory seems less likely, when the energy required for protein transport from the tubers to the seedpods is considered. It seems more likely, that the final degradation of tuber storage proteins results in the completion of the formation of the daughter tuber. Further research involving radiolabelled tuber protein subunits may help to verify this theory. Alternately, immunological testing of the seed proteins with antibodies raised against the tuber proteins may help to identify any relationship between the two sets of storage proteins.

The results of this study also verify that *Sandersonia* major tuber proteins are in fact storage proteins under the (above mentioned) definition (section 1. 7). Although a time course of their accumulation has not been demonstrated, the tuber proteins were observed to deplete during the reproductive periods of the life cycle, meeting the storage protein definition

4. 5 Seed Light Microscopy and TEM

4. 5. 1 Light Microscopy Observations

Plate 14 helps to explain why, at 20 DAP only traces of protein are shown on (gel) plate 7, in the seed storage protein development study (section 3. 4). At 20 DAP, the immature seed only contains an empty endosperm cavity. The gel bands on plate 7, (i.e. 20 DAP) must therefore represent the 'housekeeping' proteins of the seed coat and mother tissue. Also visible on plate 14 is differentiated mother tissue that may be the early precursor to endosperm tissue.

At 40 DAP, an immature endosperm 'ball', partly occupied the endosperm cavity (plate 15). The increase in storage protein stored in this 'ball' is responsible for the rapid increase in total soluble seed protein (figure 4), from 20 to 40 DAP. At 40 DAP, the storage proteins present in the immature endosperm 'ball' displayed the characteristic 6 subunit (band) pattern (Plate 7). It also appears that the immature endosperm 'ball' begins to form at approximately 30 DAP, the earliest time the 6 storage proteins bands were detectable.

At 51 DAP, the endosperm 'ball' has almost completely filled the endosperm cavity and that an embryo has formed (Plate 17). This correlates with the period when the fresh and dry weights reach a maximum (figures 1 and 2).

At 62 DAP, the endosperm cavity has filled completely (plate 18). At this time the whole seed is beginning to desiccate (figures 1, 2 and 3). Compared to plate 17, an enlarged embryo is visible in plate 18.

4. 5. 2 TEM

4. 5. 2. 1 Protein Body Observations

Previous research has found that protein bodies vary in structure between plant families but show a resemblance within families. Lott (1978) suggests a series of 11 models of protein body structure, differing in the variety of constituents. The protein bodies present in *Sandersonia* seeds fit into two of the classes suggested by Lott (1978) and one other combination not included in the 11 models.

Found at a variety of seed ages, one protein body type contains only proteinaceous matrix material, of varying 'graininess'. These structures do not contain any inclusions, are quite common between the sampled seed ages and vary in size, from small (2 μm) to large (>20 μm).

A second type of protein body contains matrix material and electron dense, black globoid inclusions. This structural type is rare (visible on plates 23 A and B) and physically small (~1-2 μm), when compared to the size of the other types.

The third structural type of protein body present is similar in size and frequency to the first type, but contain up to ten soft globoid inclusions (plate 31 A). The structure and function of soft globoid inclusions is still an area of debate. Soft globoids may be spaces in which liquid is stored, or cavities remaining unfilled since protein body formation. It is also unclear whether the soft globoids (as well as the crystal globoids) are a source of phytic acid (Werker, 1997).

Plates 19-26 also confirm the lack of any crystalloid protein body inclusions or druse crystals within *Sandersonia* seed protein bodies. This confirms the SDS PAGE evidence presented in section 4.2 (plate 3).

The first two structural types described in *Sandersonia* seeds are described by Lott (1978), though the third type is not. This is not surprising, considering the variety of structures possible. The phenomenon of having more than one structural protein body type is also shared by other plant species. *Iris pseudoacorus* contains 5 different structural types (Werker, 1997).

Lott (1978) described protein bodies as a possible tool in plant systematics, acknowledging the similarity of protein bodies of plants within the same families. At the time of Lott's (1978) work, research relating to protein body structure and inclusions covered only a restricted range of crop and food related plants (Briarty *et al*, 1969; Craig *et al*, 1979; Craig *et al*, 1980; Pernollet, 1978).

Recently, the protein body structure of a slightly larger range of non-crop plants has been investigated. Based on protein body structure (commonly containing soft globoid inclusions), *Sandersonia* is grouped together with a range of other flowering plants; *Aellenia austrani*, *Lupinus albus*, *Linum* (family), *Iris* spp and *Cucumis* (family) (Werker, 1997). As the closest relatives to *Sandersonia* (regarding protein body structure) are from diverse taxonomic groups, the value of protein body structure in systematic study is debatable. It would be interesting to examine the protein bodies of *Sandersonia*'s closest relatives, *Littonia* and *Gloriosa*, to identify any family similarity.

Light microscopy observations and SDS PAGE results infer that the endosperm formation in *Sandersonia* seeds begins at approximately 30 DAP. Before this time, at 20 DAP, only small numbers of protein bodies are visible in the

seed mother tissue cells. These partially filled protein bodies are separated from the cytoplasm by membranes and occupy the region described in section 4.5.1 as (possibly differentiated) mother tissue. By 40 DAP the accumulation of protein bodies has started. At this time there was differentiation in the numbers and sizes of protein bodies between the inner and outer endosperm tissue. Differentiation in the protein body numbers of different tissues in other plants has been widely noted in other reports (Pernollet, 1978, Lott, 1978). The inner endosperm cells contain a greater number of protein bodies compared to the outer endosperm cells. Soft globoid inclusion bodies are visible in the protein bodies at 40 DAP. Until maturity (greater than 60 DAP), the numbers and sizes of protein bodies, increase in the inner and outer endosperm cells.

4. 6. 2. 2 Possible Functions of the Thickened Endosperm Cell Walls

During the protein body investigation, the relative thickness of the endosperm tissue cell walls was noted. When compared to the cell wall thickness of seed coat cells (non-differentiated mother tissue), the endosperm cell walls are massively enlarged. The enlarged cell walls were visible at 20 DAP in the differentiated mother tissue (plate 27 B). At this time the cell walls appear to have formed completely in relation to width, but are only partially complete in relation to the deposition of waxes. At 40 DAP the cell walls have the appearance of mature cell walls (>60 DAP) regarding wax formation, though intercellular spaces are still present. At seed maturity the intercellular spaces seem to have disappeared.

The enlargement of endosperm cell walls can fulfil several different functions. In one sense, this observation is associated with the desiccation process at seed maturity. According to Raven *et al* (1992) the 'stripe' visible on plate 29 is alternate layers of wax (cutin and suberin), deposited for cellular protection during desiccation. With this function in mind, the suggested function of the soft globoid inclusions (as a store of fluids, Werker, 1997) is partially verified. During seed desiccation, at maturity, the cell wall waxes may prevent any extra water loss. During this period, the soft globoids may provide water during desiccation for

cellular housekeeping functions or as an extra source of water for rapid cell reanimation during germination.

Endosperm cell wall thickening in *Sandersonia* seeds also coincides with a lack of visible starch granules or starch amyloplasts in those cells. At each of the sampled seed ages, starch granules are only visible in the outer non-endosperm seed mother tissues (section 3. 10. 2. 2 and plate 29 A). This suggests a second possible function of the enlarged endosperm cell walls is one of carbohydrate storage. The most common form of carbohydrate storage in seeds is starch. A smaller proportion of plants store polysaccharides in their cell walls (Fry, 1989).

Thickened cell walls and an absence of starch granules are also observed in the seed tissues of *Capsicum annuum* (Chen and Lott, 1991), *Myrsine laetevirens* (Otegui *et al*, 1998), date palm (*Phoenix dactylifera*) (DeMason *et al*, 1989) and fenugreek (*Trigonella foenum-graecum*) (Meier and Reid, 1977). Cell wall utilising plants can be further subdivided into a range of groups relating to the type of carbohydrate stored. From the above studies, fenugreek and date palm seeds deposit galactomannans, *Capsicum annuum* stores mannan containing polysaccharides and *Myrsine laetevirens* stores xyloglycans. Although each of these storage polysaccharides has a similar function; that of sustaining the carbohydrate requirements of the germinating seed, there appears to be a different mode of deposition of each. Galactomannans are formed in the intra-cisternal space of the RER then released outside the plasmalemma, whereas the deposition of other storage polysaccharides is mediated by golgi vesicles (Meier and Reid, 1977).

Further research, to establish the type of storage polysaccharide present, is still required. This would involve biochemical analysis as well as differential staining of microtomed seed sections for subsequent observation with light and epifluorescence microscopy

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APPENDIX 1

1.1 Buffer Solutions and Reagents

A. Phosphate Buffer

Solution 1. Potassium dihydrogen phosphate (BDH Laboratory Supplies, Poole, England) 13.61 g/L

Solution 2. Disodium hydrogen phosphate (BDH Laboratory Supplies, Poole, England) 35.81 g/L

Solution 1 (400 mL) was combined with solution 2 (600 mL) to make 1 L.

B. Sample Buffer

Distilled Water 3.2 mL

1.0 M Tris-HCl pH 6.8 1.0 mL

Glycerol (BDH Laboratory Supplies, Poole, England) 1.6 mL

10% SDS (w/v) 1.6 mL

2-B-mercaptoethanol (BDH Laboratory Supplies, Poole, England) 0.4 mL

All the compounds were combined except glycerol and mercaptoethanol, which were added dropwise until mixed. The buffer was stored in eppendorf tubes at 4°C.

C. Crystalloid Protein Extraction (CPE) Buffer

(containing 65mM Tris-HCl pH 6.8, 2% (w/v) SDS and 10% (w/v) glycerol from Gifford *et al* (1982).

Distilled Water 85.6 mL

1 M Tris-HCl pH 6.8 6.5 mL

SDS (BDH Laboratory Supplies, Poole, England) 2 g

Glycerol (BDH Laboratory Supplies, Poole, England) 7.9 mL
The SDS, glycerol and Tris-HCl were dissolved in half the final volume (100 mL).
The pH was tested and the solution then brought up to the final volume with distilled water.
Whenever used, crystalloid protein extraction buffer was diluted to a 50:50 mixture with phosphate buffer.

1.2 Protein Determination

1.2.1 Bradford Reagent

Coomassie Brilliant Blue G-250 (SIGMA® Chemical Company) 100 mg
Ethanol 95%(v/v) 50 mL
Phosphoric Acid 85%(w/v)(BDH Laboratory Supplies, Poole, England) 100 mL

Coomassie Brilliant blue G-250 was dissolved in ethanol. Phosphoric acid was added then the volume was diluted to 1 L with distilled water. The solution was filtered then left to stabilize for three days. The reagent was stored in a brown bottle.

1.2.2 Bradford Assay Standard Curve Preparation

A range of protein concentrations, 0-100µg/mL, was prepared by diluting a stock solution of Bovine Serum Albumin (BSA)(10mg/mL) with distilled water. The known protein samples (100 µL) were fixed with Bradford reagent (1 mL), mechanically mixed (by vortex) and the absorbance noted at 595 nm. A standard curve was prepared, and the equation of the trend line established using Microsoft Excel. All Bradford assays carried out used the same bottle of reagent and calculated standard curve.

1.3 Reagents and Gel Preparations for SDS PAGE

(Laemmli Buffer System (Laemmli 1970))

1.3.1 Stock Solutions

A. SDS PAGE Sample Buffer

Distilled Water	3.2 mL
1.0 M Tris-HCl pH 6.8	1.0 mL
Glycerol (BDH Laboratory Supplies, Poole, England)	1.6 mL
10% SDS (w/v)	1.6 mL
2-B-Mercaptoethanol (BDH Laboratory Supplies, Poole, England)	0.4 mL
0.1% Bromophenol Blue (w/v)	0.2 mL

The distilled water, Tris-HCl, SDS and bromophenol were mixed in a vial. Glycerol and mercaptoethanol were added dropwise. The buffer was stored in eppendorf tubes at 4°C

B. 1.0 M Tris-HCl pH 6.8

Distilled Water	250 mL
Trizma Base (SIGMA® Chemical Company)	12.114 g

The Trizma Base was dissolved in half the quantity of distilled water and adjusted to pH 6.8 by concentrated HCl added drop wise. The solution was then made up to the final volume and stored in a glass bottle at 4°C.

C. 1.0 M Tris-HCl pH 8.8

Distilled Water	250 mL
Trizma Base (SIGMA® Chemical Company)	30.285 g

The Trizma base was dissolved in half the quantity of distilled water, the pH adjusted to 8.8 and the solution made up to the final volume. The solution was stored in a glass bottle at 4°C.

D. 10% SDS (w/v)

Distilled Water	100 mL
SDS (BDH Laboratory Supplies, Poole, England)	10 g

The SDS was dissolved in distilled water and stored in a glass bottle at room temperature to avoid precipitation. When the solution was required, any precipitate present was dissolved by heating the storage bottle in a 37°C water bath.

E. 30% Acrylamide/Bis w/v

Acrylamide (BDH Laboratory Supplies, Poole, England)	29.2 g
Bis (SIGMA® Chemical Company)	0.8 g

The acrylamide and Bis were dissolved in distilled water and made up to 100 mL. The solution was filter through filter paper and stored at 4°C.

F. 10% Ammonium Persulphate (w/v)

Distilled Water	200 µL
Ammonium Persulphate (Bio-Rad Laboratories, CA, USA)	20 mg

The ammonium persulphate was dissolved in 200µL distilled water. The solution was stored in an eppendorf tube at 4°C for no longer than seven days.

G. 0.1 % Bromophenol Blue (w/v)

Distilled Water	5 mL
Bromophenol Blue (May and Baker LTD, Dagenham, England)	5 mg

The Bromophenol Blue was dissolved in distilled water and made up to the final volume. The dye solution was stored at room temperature.

H. Coomassie Stain

Distilled Water	200 mL
100% Methanol	160 mL
Acetic Acid (BDH Laboratory Supplies, Poole, England)	40 mL
Coomassie Brilliant Blue R-250 (SIGMA® Chemical Company)	0.4 g

The solutions were combined and stored in a dark bottle at room temperature. The stain was recycled after each staining run and reused. Gels were stained for thirty minutes in ice cream containers on a rotary agitator.

I. Destain

Distilled Water	1000 mL
100% Methanol	800 mL
Acetic Acid (BDH Laboratory Supplies, Poole, England)	200 mL

The solutions were combined and stored in a dark bottle at room temperature. Gels were destained and stored in destain solution with one change after thirty minutes.

J. SDS PAGE Running Buffer
(5X Running Buffer Stock)

Trizma Base (SIGMA® Chemical Company)	7.5 g
Glycine (SIGMA® Chemical Company)	36 g
SDS (BDH Laboratory Supplies, Poole, England)	2.5 g

All the compounds were combined and dissolved in distilled water. The solution was made up to 500 mL and stored in a plastic bottle at room temperature. During individual gel runs, 60 mL 5X running buffer was diluted with 240 mL distilled water.

1.3.2 SDS PAGE Gel Preparation

A. SDS PAGE Separating Gel 12%

Distilled water	1.675 ml
1.0 M Tris-HCl pH 8.8	1.25 ml
10% SDS w/v	50 µl
30% Acrylamide/Bis w/v	2.0 ml
10% Ammonium persulphate w/v	25 µl
TEMED (Bio-Rad Laboratories, CA, USA)	2.5 µl
Total monomer	5 ml

B. SDS PAGE Separating Gel 15%

Distilled water	0.5 ml
1.0 M Tris-HCl pH 8.8	1.875 ml
10% SDS w/v	50 µl

30% Acrylamide/Bis w/v	2.5 ml
10% Ammonium Persulphate w/v	50 μ l
TEMED (Bio-Rad Laboratories, CA, USA)	2.5 μ l
Total Monomer	5 ml

C. SDS PAGE Stacking Gel 4%

Distilled water	3.05 ml
1.0 M Tris-HCl pH 6.8	1.25 ml
10% SDS w/v	50 μ l
30% Acrylamide/Bis w/v	0.65 ml
10% Ammonium Persulphate w/v	25 μ l
TEMED (Bio-Rad Laboratories, CA, USA)	5 μ l
Total Monomer	5 ml

In each of the three listed gels, ammonium persulphate was prepared within 7 days of gel preparation.

1.3.3 SDS PAGE Protein Standard Preparation

SDS PAGE Molecular Weight Standards, Broad or Low Range (Bio-Rad Laboratories, CA, USA)	2 μ L
SDS PAGE Sample Buffer	38 μ L

The molecular weight standard (broad or low range) was diluted 1:20 in buffer and vortexed in a labelled eppendorf tube. The lid was punctured and the standard incubated at 100°C for 5 minutes by water bath. The standard was stored at -20°C.

1.4 TEM and Light Microscopy Sample Preparation

1.4.1 Stock Solutions

A. 0.2 M Buffer A

Distilled water	500 mL
Disodium hydrogen orthophosphate dihydrate (GPR™) (BDH Chemicals, Poole, England)	17.8 g

The Di-sodium hydrogen orthophosphate di-hydrate was dissolved in distilled water and stored at room temperature.

B. 0.2 M Buffer B

Distilled water	500 mL
Sodium dihydrogen orthophosphate dihydrate (GPR™) (BDH Chemicals, Poole, England)	15.6 g

The Sodium dihydrogen orthophosphate dihydrate was dissolved in distilled water and stored at room temperature.

C. 0.075 M PO₄ Infiltration Buffer pH 7.2 containing 3% glutaraldehyde

Distilled water	83 mL
Buffer A	36 mL
Buffer B	14 mL
25% Glutaraldehyde solution	12 mL

Distilled water, buffer A and buffer B were mixed together in a glass bottle. 12 mL of 25% glutaraldehyde solution was added to 88 mL of infiltration buffer. The remaining quantity of infiltration buffer prepared each session was used in post-fixation and post-osmium rinsing steps.

D. Araldite composition

Part A Epoxy Resin

Part B Epoxy Hardener

A 50:50 mixture of the two parts was prepared on a dish and used immediately.

1.5 Potting Mix/Vermiculite Constituents

A. Potting Mix Constituents

Bark	60%
Peat	20%
Sterilized Soil	10%
Sand	10%

Also contains 8-9 month release Nuticote and micronutrients.

(Produced by A. Torrance, Halswell Junction Road, Christchurch)

B. Vermiculite

Medium grade vermiculite from Nuplex Industries LTD, Penrose, Auckland was used.

APPENDIX 2

At the time of writing this thesis, the Internet has become a prominent tool in information 'promotion' and gathering. Though it is recognised that the information obtained via the Internet is not subject to peer review, as in the primary literature, it is of superficial use, pending further verification. The number of Internet web sites relating to *Sandersonia* give an indication of the commercial importance of the species.

The following list of World Wide Web (Internet) addresses represent a small number of New Zealand companies that, at the time of writing this thesis, were actively promoting the availability and sale of *Sandersonia* (*Sandersonia aurantiaca*) tubers and cut stems, domestically and internationally;

www.flownz.co.nz

www.bloomz.co.nz

www.pharetuber.co.nz

www.mysite.xtra.co.nz/~oceanz/

www.freshcut.co.nz.....marketers for Tuberland.

www.franjogardens.co.nz

www.multiflora.co.nz

www.greenhouse.co.nz

Information on the commercial production of *Sandersonia* tubers is available from:

www.croplink.co.nz

www.crop.cri.nz/broadsheet/sanders.htm

www.irl.cri.nz

www.tarweed.com

The second Internet site listed (crop and food research, a crown research institute), is the source of information in this thesis, referred to by footnote as Clark (1994).

It is only over the Internet that the most extravagant claim regarding Sandersonia are made. Multiflora Ltd claim to have produced a yellow variety of Sandersonia, known as 'Lutea'. A primary literature search has not revealed any publications relating to this discovery. As another measure of the commercial interest relating to Sandersonia production, greenhouse.co.nz and as early as 1993, Floraculture NZ Ltd, have advertised the licensing of potential growers. These companies also offer an inclusive package involving tunnel house and infrastructure construction, for the perspective novice grower.